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PRINCIPAL INVESTIGATOR: Peter Hornsby, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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Peter Hornsby, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

e-mail:
phornsby@bcm.tmc.edu

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13. ABSTRACT ***(Maximum 200 Words)***

These experiments investigate a mouse model for the biosynthesis of the human adrenal androgens (dehydroepiandrosterone, DHEA, and its sulfate, DHEAS) and the role of these steroids in human breast cancer growth. An androgen-dependent human breast cancer model was established in immunodeficient (*scid)* mice. Zona reticularis cells in the human adrenal cortex are responsible for adrenal androgen biosynthesis because of the suppressed expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) in these cells. A protein present in the non-DHEA-secreting zones of the cortex and absent from the zona reticularis which binds to a regulatory region of the type II 3β-HSD gene was partially purified. Human adrenocortical cells were transplanted into *scid* mice and were shown to replace the animals' own adrenal function. Although zona reticularis cells were transplanted, DHEAS was not detected in mouse plasma. As an alternative to the use of human zona reticularis cells, clonal bovine adrenocortical cells were shown to be capable of forming tissue in *scid* mice that replaces the animals' adrenal glands. This was shown both with normal clonal cells and with cells genetically modified by the insertion of marker genes. The ability to genetically modify the cells provides a means to test whether suppression of 3β-HSD by an antisense strategy can create a tissue with a very high rate of DHEA biosynthesis in the mouse transplant model.

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Peter Hinsley 9/29/99

PI - Signature Date

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Introduction

Unlike human cancers which are clearly correlated with readily identifiable aspects of lifestyle, for example lung cancer and smoking history, breast cancer does not appear to be dependent on exposure to specific environmental carcinogens. Rather, the major risk factor for breast cancer in women appears to be the lifetime exposure of breast tissue to circulating and local natural estrogens and androgens. Estrogens of various types can act both as co-initiators of carcinogenesis (together with other agents -- possibly universally present carcinogens) and as growth factors for breast cancer tissue, particularly in a subset of tumors which are strongly dependent on estrogen for growth. Estrogens to which the breast tissue is exposed are of both ovarian and extra-ovarian origin. In both men and women, the major source of tissue estrogens are circulating androgens and androgen precursors which are locally converted via aromatase to an active estrogen. The major circulating androgen precursor is dehydroepiandrosterone (DHEA) and its sulfate, DHEAS. These androgen precursors originate overwhelmingly from the adrenal cortex in women rather than from the ovary. Surprisingly, it has also recently been shown that even testosterone in the ovarian follicle itself can be formed from circulating DHEAS of adrenocortical origin. For these reasons, it is clear that production of DHEAS and other androgens by the adrenal cortex forms a major lifetime risk factor for breast cancer development in women. From the early part of this century it was known that adrenalectomy improves survival in a substantial fraction of women with breast cancer. Although no longer performed, because of an unacceptable incidence of complications, adrenalectomy was effective because it removed the source of adrenal androgens and deprived the breast cancer of its major source of estrogens. The aim of the studies here is to provide basic information on the regulation of androgen precursor synthesis by the human adrenal cortex and to test the effects of adrenal androgen synthesis on human breast cancer growth in a mouse model. The immunodeficient *scid* mouse is used both as host for functional human adrenal organoids as a source of androgens and has human breast cancer cells implanted as a target tissue. Uses of the information obtained on human adrenocortical DHEAS production are the identification of hormonal and molecular factors that set the adrenal androgen production level. This information may more precisely define the risk factors of adolescent and postadolescent women for higher peak levels of DHEAS and consequent increased exposure of the breast tissue to estrogens. The better characterization of the factors that regulate adrenal androgen synthesis, currently very poorly defined both molecularly and physiologically, would enable appropriate diagnosis and interventions in high-risk women and may provide other avenues of rational treatment in estrogen-responsive breast cancer.

Body

The aims of the funded grant are to set up a mouse model for human adrenal androgen production by the adrenal cortex and the effects of these androgens on the growth of human breast cancer.

The specific tasks were as follows:

1. Further develop the human adrenal organoid/*scid* (severe combined immunodeficiency) mouse model for investigation of the regulation and effects of human adrenal androgens.

Progress:

An immunodeficient mouse/xenotransplant model has been set up in which human adrenocortical cells are grown as a functional tissue in the *scid* mouse (see publications Hornsby et al., 1998; Popnikolov and Hornsby, 1999 in Appendix). The human adrenal cells, now vascularized and forming a functional tissue structure, replace the animal's own adrenal glands and secrete steroids which maintain its health and life. This is the first time that a mouse endocrine organ has been replaced entirely in its function by transplanted human cells. This humanized mouse model can be used to study aspects of adrenal physiology, cell biology

and biochemistry, which would be impossible in intact human subjects (Hornsby, 1999a). Our specific aim with respect to this grant was to establish whether these transplanted tissues secrete the adrenal androgens dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS).

Problems:

During the period of this grant we made the discovery that DHEA and DHEAS are secreted exclusively from the zona reticularis cells of the adrenal cortex (Endoh et al., 1996). These cells form the innermost zone in the adult human adrenal cortex. On the other hand, the zona fasciculata cells of the human adrenal cortex, which form the middle zone, secrete the glucocorticoid cortisol and do not secrete adrenal androgens. We prepared these two cell types separately from the human adrenal cortex and transplanted them into *scid* mice. Both cell types formed functional tissues, but, in both cases, the tissues secreted cortisol without detectable DHEAS in the mouse plasma (Thomas et al., 1997b Hornsby et al., 1998). This could indicate that the cells lost their ability to make DHEA. However, there are other possibilities to consider. The first possibility is that the cells re-differentiate after transplantation and that they tend to differentiate into zona fasciculata cells, rather than zona reticularis cells. This indicates a need for more understanding of the way in which the zonation of the adrenal cortex is set up and how this differentiation is regulated at the molecular level. This aspect of the research is addressed in a further section. The second possibility is that in mice DHEAS may not achieve the high circulating levels that it does in humans, even if DHEA is entering the circulation. We did experiments in which DHEA was administered to mice in their drinking water. Plasma DHEAS levels did not rise to detectable levels. Thus, there are extra-adrenal differences between mouse and human physiology which will need to be addressed before the mouse can be used as a totally reliable model of human adrenal physiology. However, the experiments performed under this grant provide the basis for future studies. Human adrenal tissue has been established in mice, and the data thus far indicate a need to understand the basis of the functional differentiation of the cells into the zones of the human adrenal cortex. Even if DHEAS levels do not rise in mouse plasma, there may nevertheless be androgenic effects of DHEA secreted by the transplanted cells on target mouse tissues. In experiments done by others, in which DHEA was administered to rodents, androgenic effects on target tissues such as the prostate were clearly seen.

Other experiments have been designed to investigate if zonation can be regulated in the transplanted cells by altering the protocol for transplantation. We developed polymer foams formed by modification of poly(L-lactic acid) to enable covalent binding of biopolymers (Zheng et al., 1998). These foams can be used for high-density three-dimensional culture of adrenocortical cells, and we thought it was possible that they might support a larger scale transplant structure in vivo. However, they did not support adrenocortical cell growth in the transplant model (Hornsby et al., 1998). Other ways to control the zonation of the transplant tissue are needed, as described below.

We also considered the possibility that cell death in the first few days after cell transplantation might affect the outcome of the experiments, for example if zona reticularis cells were more likely than zona fasciculata cells to die before a vascularized tissue structure is achieved. We set up a model for these early events using bovine adrenocortical cells (Tunstead et al., 1999). The data show that vascularization (invasion of endothelial cells) is the most important influence on cell survival and growth. Better understanding of the factors that control vascularization would help in achieving a transplant tissue with functional zona reticularis cells.

<u>2. Assess the influence of circulating adrenal androgens on human estrogen-responsive human breast cancer cell growth.</u>

Progress:

To test the effect of human adrenal androgens on growth of estrogen-responsive breast cancer, we have developed growth of human breast cancer in *scid* mice in an androgen-dependent manner (Thomas et al., 1997b). MCF-7 cells, a well-studied human breast cancer cell line, were transfected with aromatase cDNA, thus mimicking the normal situation of the human breast, which is exposed to estrogens derived from circulating androgens via stromal tissue aromatase. We showed that these cells can grow as an androgen-dependent transplantable tumor line.

Problems:

Further progress is not possible until transplants are achieved that have functional zona reticularis cells. Future studies will be designed to assess the effect of androgens secreted by transplanted adrenal cells on tumor growth.

3. Investigate the molecular biology of adrenal androgen regulation, focusing on the key enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD).

Progress:

This task requires the transplantation of genetically modified adrenocortical cells in the mouse model. This was explicitly proposed in the grant application, but not all of the procedures necessary to realize this aim were in place at that time; many of them have been developed during the period this grant. At this point, we are a much better position to directly test the results of genetic modification on the function of human adrenal cells in the mouse model. The following progress has been made:

First, we formed clonal tissue from normal (not genetically modified) clonal bovine adrenocortical cells (Thomas et al., 1997a).

The ability to form tissue from clonal cells is a necessary step for the production of tissue from genetically modified cells, because the selection of transfected cells in culture which is required for genetic modification produces clonal populations of cells. Therefore we reasoned that the production of tissue from normal clonal cells would be a pre-requisite for the production of tissue from genetically modified clonal cells.

For bovine cells, we showed that xenotransplanted adrenocortical tissue of clonal origin can be formed in *scid* mice by using techniques of cell transplantation (Thomas et al., 1997a). We studied in detail a single clone of bovine adrenocortical cells, but 5 of 20 other randomly selected clones also formed tissue. Most adrenalectomized animals bearing transplanted cells survived indefinitely, demonstrating that the cells restored the animals' capacity to survive in the absence of sodium supplementation. Formation of well-vascularized tissue at the site of transplantation was associated with stable levels of cortisol in the blood, replacing the mouse glucocorticoid (corticosterone). Ultrastructurally, the cultured cells before transplantation had characteristics of rapidly growing cells, but tissue formed *in vivo* showed features associated with active steroidogenesis. These experiments show that an endocrine tissue, functionally replacing the corresponding tissue of the host animal, can be derived from a single normal somatic cell.

Moreover, for bovine adrenal cells, we have produced genetically modified tissue, which is functional and replaces the normal adrenal function of the animals. This is the first time that a xenotransplanted genetically modified tissue has been used to replace the essential function of one of an animal's organs. The genes that were used to modify the cells have been both "marker" genes and genes that change the properties of the cells (telomerase reverse transcriptase) (Thomas et al., 1999). We have also very recently transplanted genetically modified human adrenocortical cells, in which the telomerase reverse transcriptase gene was introduced by a defective retrovirus.

Problems:

Attempts to form tissue from clonal human adrenocortical cells have been unsuccessful (Hornsby et al., 1998). Nevertheless, we believe that these difficulties are predominantly technical in nature and we expect that we will be able to perform the same kinds of experiments on human adrenocortical cells that we have performed on bovine cells. The recent demonstration that human adrenocortical cells can be genetically modified by retroviral transduction shows that construction of genetically modified but not necessarily clonal human adrenal organoids may be possible.

4. Assess physiological influences on adrenal androgen production in the human adrenal organoid/*scid* mouse model.

Progress:

There is a great lack of knowledge of the factors that influence the development of zona reticularis cells and their hormonal regulation (reviewed in Hornsby, 1997b).

Problems:

Further progress is not possible until transplants are achieved that have functional zona reticularis cells. Future studies will be designed to assess the effect of factors on androgen secretion by transplanted adrenal cells.

5. Identify transcription factors which regulate the human type II 3β-HSD gene and test their effects on adrenal androgen synthesis in the human organoid/*scid* mouse model.

Progress:

A major area we have studied under this grant is the nature of the transcription factors which regulate the 3β-HSD gene and how its level of expression is suppressed in the zona reticularis. We began this work by observing that the differences between the type I and type II human 3β-HSD genes are slight. They are very similar in their promoter and regulatory elements, yet the type I gene is expressed in most tissues throughout the body and the type II gene is expressed only in steroidogenic tissues; specifically, within the adrenal cortex, only in the zona glomerulosa and the zona fasciculata. In examining the differences between the genes, one key area in the first intron came to our attention. We decided to examine if there are proteins in the adrenal cortex which bind specifically to this sequence. This resulted in the identification of a DNA-binding protein present in the human zona fasciculata, but not in the zona reticularis, and also present at high concentrations in the bovine adrenal cortex (manuscript in preparation). This was shown by gelshift assay and Southwestern blotting. We have partially purified this protein from the bovine adrenal cortex. In the first steps of the process the protein has been separated on a DEAE column with a ~30-fold enrichment as judged by gelshift assay. In a second step, the protein was purified by binding to a biotinylated oligonucleotide attached to streptavidin magnetic beads. If this protein is, in fact, a transcriptional regulatory protein that is differentially expressed between the zones, it will be the first example of such a protein. No transcription factors which differ in levels between the zones and thus regulate the relative production of mineralocorticoids, glucocorticoids, and androgens have yet been identified.

Problems:

We do not yet have a sufficiently pure protein preparation for sequence analysis. If the protein is purified sufficiently it can be subjected to micro amino acid sequencing for identification and cloning.

Key Research Accomplishments

o Developed mouse model for studying human adrenal function in vivo, with future application to study of adrenal androgens

o Developed methods for genetic modification of adrenocortical cells

o Showed that genetically modified adrenocortical cells may be transplanted with continued formation of functional tissue

o Demonstrated that the synthesis of the adrenal androgen, DHEA, is associated with the lack of a DNA-binding protein in the DHEA-synthesizing cells in the zona reticularis.

Reportable Outcomes

Manuscripts (included in Appendix)

Hornsby, P.J. (1997) DHEA: A biologist's perspective. *J. Am. Geriatrics Soc.* 45: 1395-1401.

Hornsby, P.J. (1999) The new science and medicine of cell transplantation. *A.S.M. (American Society for Microbiology) News* 65: 208-214.

Hornsby, P.J., Thomas, M., Northrup, S.R., Popnikolov, N.P., Wang, X., Tunstead, J.R., and Zheng, J. (1998) Cell transplantation: A tool to study adrenocortical cell biology, physiology, and senescence. *Endocr. Res.* 24: 909-918.

Popnikolov, N.K., and Hornsby, P.J. (1999) Subcutaneous transplantation of bovine and human adrenocortical cells in collagen gel in *scid* mice. *Cell Transplantation (in press).*

Thomas, M., Northrup, S.R., and Hornsby, P.J. (1997) Adrenocortical tissue formed by transplantation of normal clones of bovine adrenocortical cells in *scid* mice replaces the essential functions of the animals' adrenal glands. *Nature Med.* 3: 978-983.

Thomas, M., Popnikolov, N., Wang, W., Northrup, S.R., Chen, S., and Hornsby, P.J. (1997) Human adrenal androgens. Regulation of biosynthesis and role in estrogen-responsive breast cancer in a mouse model. *The Department of Defense Breast Cancer Research Program Meeting: Era of Hope* U.S. Government Printing Office, Washington, D.C., Vol. 2: 791-792.

Thomas, M., and Hornsby, P.J. (1999) Primary bovine adrenocortical cells transplanted into *scid* mice prevent adrenal insufficiency and form vascularized functional tissue. *Mol. Cell. Endocrinol.* 153: 125-136

Thomas, M., Yang, L., and Hornsby, P.J. (1999) Formation of normal functional tissue from transplanted adrenocortical cells expressing telomerase reverse transcriptase (TERT). *(submitted).*

Tunstead, J.R., Thomas, M., and Hornsby, P.J. (1999) Early events in the formation of a tissue structure from dispersed bovine adrenocortical cells following transplantation into *scid* mice. *J. Mol. Med. (in press).*

Zheng, J., Northrup, S.R., and Hornsby, P.J. (1998) Modification of materials formed from poly(L-lactic acid) to enable covalent binding of biopolymers: Application to high-density three-dimensional cell culture in foams with attached collagen. *In Vitro Cell. Dev. Biol.* 34: 679-684.

Patent applied for: Materials formed from poly(L-lactic acid) to enable covalent binding of biopolymers.

Conclusions

The *scid* mouse/human adrenocortical tissue model has been set up and has been shown to be useful for studying human adrenal physiology, cell biology, and molecular biology. The development of clonal adrenocortical tissues in the *scid* mouse, which can also be genetically modified, has created the possibility of using genetically modified bovine adrenocortical cells as a model for adrenal androgen-producing cells in the mouse. Progress in identifying transcription factors regulating the key enzyme of adrenal androgen biosynthesis, 3β-hydroxysteroid dehydrogenase, will allow the study of adrenocortical cell differentiation and zonation, and thus the basis for androgen biosynthesis by the human adrenal cortex. The development of MCF-7 cells as an androgen-sensitive transplantable tumor line will allow the

action of human adrenal androgens secreted by adrenocortical tissue in the *scid* mouse on human breast cancer growth.

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Endoh, A., Kristiansen, S.B., Casson, P.R., Buster, J.E., and Hornsby, P.J. (1996) The zona reticularis is the site of biosynthesis of dehydroepiandrosterone and dehydroepiandrosterone sulfate in the adult human adrenal cortex resulting from its low expression of 3β-hydroxysteroid dehydrogenase. *J. Clin. Endocrinol. Metab.* 81: 3558-3565.

Hornsby, P.J. (1997) DHEA: A biologist's perspective. *J. Am. Geriatrics Soc.* 45: 1395-1401.

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Hornsby, P.J., Thomas, M., Northrup, S.R., Popnikolov, N.P., Wang, X., Tunstead, J.R., and Zheng, J. (1998) Cell transplantation: A tool to study adrenocortical cell biology, physiology, and senescence. *Endocr. Res.* 24: 909-918.

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Thomas, M., Yang, L., and Hornsby, P.J. (1999) Formation of normal functional tissue from transplanted adrenocortical cells expressing telomerase reverse transcriptase (TERT). *(submitted).*

Tunstead, J.R., Thomas, M., and Hornsby, P.J. (1999) Early events in the formation of a tissue structure from dispersed bovine adrenocortical cells following transplantation into *scid* mice. *J. Mol. Med. (in press).*

Zheng, J., Northrup, S.R., and Hornsby, P.J. (1998) Modification of materials formed from poly(L-lactic acid) to enable covalent binding of biopolymers: Application to high-density three-dimensional cell culture in foams with attached collagen. *In Vitro Cell. Dev. Biol.* 34: 679-684.

Bibliography of all publications: See above under "Reportable Outcomes".

Personnel receiving pay from this grant:

Peter J. Hornsby, Ph.D.

Lianqing Yang, M.D.

Vladimir V. Didenko, M.D., Ph.D.

Xiangdong Wang, M.D., Ph.D.

Michael Thomas, Ph.D.

Nikolay K. Popnikolov, M.D., Ph.D.

S. Robert Northrup, B.A.

James R. Tunstead, Ph.D.
Weiye Wang, M.D., Ph.D.
Ji Zheng, Ph.D.

ENDOCRINE RESEARCH, 24(3&4), 909-918 (1998)

CELL TRANSPLANTATION: A TOOL TO STUDY ADRENOCORTICAL CELL BIOLOGY, PHYSIOLOGY, AND SENESCENCE

Peter J. Hornsby, Michael Thomas, S. Robert Northrup, Nikolay K. Popnikolov, Xiangdong Wang, James R. Tunstead and Ji Zheng

Huffington Center on Aging and Department of Cell Biology, Baylor College of Medicine, Houston, Texas, USA

ABSTRACT

We have established a mouse model for the growth and function of bovine and human adrenocortical cells in immunodeficient animals. We used the technique of cell transplantation, in which dispersed cells are introduced into an appropriate host *in vivo* to form a functional tissue. The ability to regenerate vascularized tissue, of normal histology and ultrastructure, is an inherent property of transplanted adrenocortical cells. Steroids secreted by the transplants replace the essential functions of the animals' own adrenal glands. Successful methods of transplantation described here have in common that the adrenocortical cells are permitted to aggregate in a space or matrix that provides adequate extracellular fluid and appropriate nutrients and oxygen. The present experiments show the potential of cell transplantation as a tool for the investigation of adrenocortical cell biology, molecular biology and physiology. The complete potential of the system will become apparent as new uses of the technique are devised, particularly with respect to human adrenocortical cells and to genetically modified cells.

INTRODUCTION

The transplantation of cells rather than tissue fragments enables problems of massive necrosis of the transplanted tissue to be avoided. Similarly, cell transplantation does not involve the problems of successful anastamosis of the blood supply associated with transplantation of whole organs. Instead, the host supplies all the blood vessels and stromal elements for the new tissue. The ability

to construct tissues and organs from component cells enables questions of cell origin, interactions, turnover, and cellular aging within tissues to be addressed.

Our work in this area began as a new approach to the biology of cellular senescence. Adrenocortical cells, as they age in culture, show progressive deficits of function (1). We wanted to test if they could perform their normal differentiated functions if they were within a vascularized tissue structure *in vivo* rather than in a culture dish. A second motivation was to use transplanted human adrenocortical cells as part of an investigation of the regulation of adrenal androgen biosynthesis. An *in vivo* model for human adrenal androgen regulation is needed because laboratory and domestic animals do not make substantial amounts of adrenal androgens, thus necessitating the study of human adrenal cells and tissues (2).

Mice with the *scid* (severe combined immunodeficiency) mutation provide an excellent rejection-free environment for the growth of xenografts (3-5). As predicted from a variety of xenotransplant experiments, wild type mice promptly reject transplanted bovine adrenocortical cells (our unpublished observations).

Tissue formed from adrenocortical cells in adrenalectomized *scid* mice replaces the essential functions of the animals' own adrenal glands. Adrenalectomy of the host animal removes the source of endogenous adrenocortical steroids and causes increased production of ACTH and angiotensin II, which act as direct or indirect trophic factors and mitogens for adrenocortical cells (6). Older studies show that regeneration and function of autotransplanted adrenal tissue is inhibited if any adrenal tissue is allowed to remain *in situ* (7).

The mouse, now reconstituted with a xenotransplanted cell type, forms a model that enables aspects of physiology, cell biology and molecular biology to be investigated that would be impractical or impossible in human subjects or most non-rodent species. In the case of bovine adrenocortical cells, we have also extended these observations to normal clones of cells rather than primary cell populations prepared from the adrenal gland (8). These experiments demonstrate for the first time that an endocrine tissue, replacing the host animal's organ, can be derived from a single normal somatic cell.

Several transplantation methods have been tested (Table I). Successful transplants secrete steroids in sufficient amounts to rescue the host animals from the lethal effects of adrenalectomy and allow the animals to survive indefinitely, as described in more detail below. The secretion of steroids by the transplants was

TABLE I

Summary of methods used for transplantation of human and bovine adrenocortical cells in *scid* mice

Transplantation method	Success[a]
Subcutaneous injection of cell suspension	No
Subcutaneous transplantation of cells attached to polyester cloth	No
Injection of cell suspension under the kidney capsule	Partial
Injection of cell suspension into polycarbonate cylinder under the kidney capsule	Yes[b]
Transplantion of cell suspension in polycarbonate cylinder attached to surface of kidney	Partial
Transplantation of cell suspension in Teflon tube, various sites in the body	Partial
Subcutaneous transplantation of cells attached to porous polymer foams	No
Subcutaneous transplantation of cell suspension in collagen gel	Yes[b]
Subcutaneous transplantation of cell suspension in Matrigel	No
Subcutaneous transplantation of cell suspension in gelatin capsule	Yes[b]

[a]Success is defined as the formation of discrete vascularized adrenocortical tissue that secretes cortisol and rescues the animals from the lethal effects of adrenalectomy. In all cases bovine or human adrenocortical cells were mixed at a ratio of 5:1 with mitomycin C-treated FGF-secreting 3T3 cells (see text).
[b]Published (8) or to be published in full elsewhere.

monitored as plasma cortisol levels. Cortisol gives an unambiguous measure of the function of the transplanted cells because this steroid is not produced by the host animal. Mice lack expression of steroid 17α-hydroxylase in the adrenal cortex, thus resulting in the biosynthesis of corticosterone rather than cortisol (9, 10). Histologically and ultrastructurally, successful transplants comprise a tissue structure resembling normal adrenal cortex (8).

RESULTS

Simple Subcutaneous Transplantation of Adrenocortical Cells: We first attempted to transplant adrenocortical cells by direct injection under the skin of *scid* mice, but this was unsuccessful (Table I). We tested whether a biocompatible polymer might be needed to provide a stable anchor for the transplanted cells during

formation of a tissue. Previously, we used polyester fabric to develop a novel technique for mammalian cell replica plating and clone selection based on gene expression (11). The same material is used in human prosthetic implants, e.g. in cardiovascular surgery (12). However, cells growing on this polymer did not survive after transplantation *in vivo*.

Transplantation into the Kidney: In view of the results of these preliminary experiments, we concluded that a different site in the body would be needed for adrenocortical cell survival. The subcutaneous site likely lacks sufficient fluid to bathe the cells with nutrients and oxygen required for their survival. In contrast, the extracellular environment within organs such as the kidney is fluid-rich and has been shown to support survival of transplanted tissues and cells (13-15).

We also hypothesized that a critical step in the long-term survival and function of the cells would be their ability to become vascularized. The state of an aggregate of transplanted cells is similar to that of a small malignant tumor; unless it achieves vascularization it will die or remain very small, limited by the radius of diffusion of oxygen from existing capillaries (16). Therefore, to promote angiogenesis in the transplanted cells, we supplied a source of a potent angiogenic factor. Adrenal cells were co-transplanted with a 3T3 cell line engineered to produce a secreted form of acidic FGF (17). These cells were rendered incapable of division by mitomycin C treatment.

When bovine adrenocortical cells were directly injected under the kidney capsule they formed functional nodules (Figure 1) but it was difficult to obtain adrenocortical tissue of a defined shape and size. The number and size of the nodules formed was highly variable.

For these reasons, we designed a small polycarbonate cylinder to confine the cells after injection, while retaining the advantages of the use of the subrenal capsule site for transplantation. This method was used in our experiments on the transplantation of clonal bovine adrenocortical cells (8) and the same technology has been applied to primary bovine adrenocortical cells and to human adrenocortical cells. A summary of the results of these experiments is presented in Table II. The reason for the failure of some bovine adrenocortical cell clones, and all tested human adrenocortical cell clones, is not yet known.

Although animals have usually been sacrificed at 30 to 40 days following cell transplantation, a small number of animals bearing transplanted primary bovine

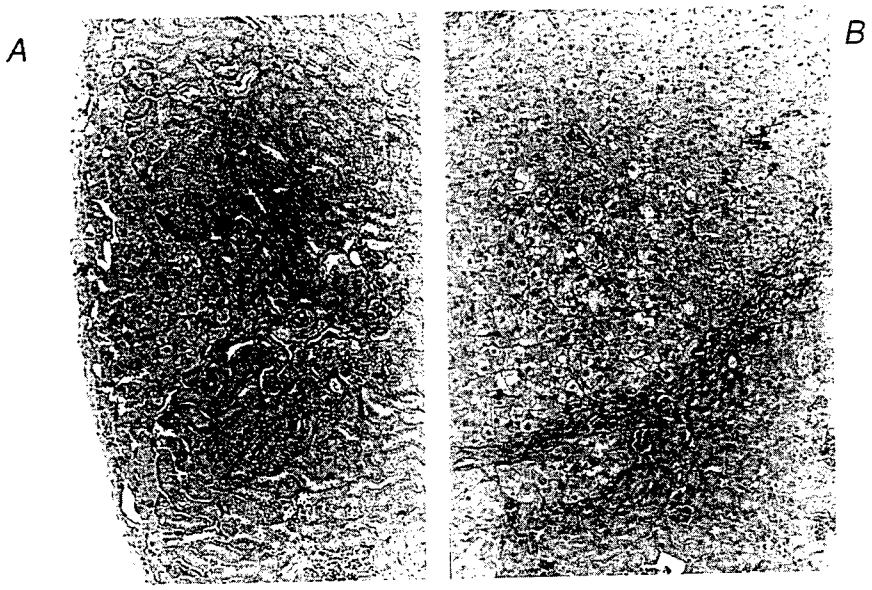

FIGURE 1

Histological appearance of nodules of bovine adrenocortical tissue formed by direct injection of cells beneath the kidney capsule in *scid* mice. ***A*** shows a shallow nodule; note the juxtaposition of the cells with the kidney parenchyma. No signs of inflammation or rejection are seen. ***B*** shows the structure of a large nodule penetrating deep into the kidney.

TABLE II

Summary of results of transplantation of adrenocortical cells beneath the kidney capsule

Type of cell	Succesful transplantation
Primary bovine adrenocortical cells	>90%[a]
Primary human adrenocortical cells	>90%[a]
Clonal bovine adrenocortical cells	~25% (all clones)[a]
	>90% (clone 14)[a]
	~0 (clone 5)[b]
Clonal human adrenocortical cells	~0[b]

[a]Published (8) or to be published in full elsewhere; [b]unpublished observations (clone 5 was derived at the same time as clone 14; see ref. 18).

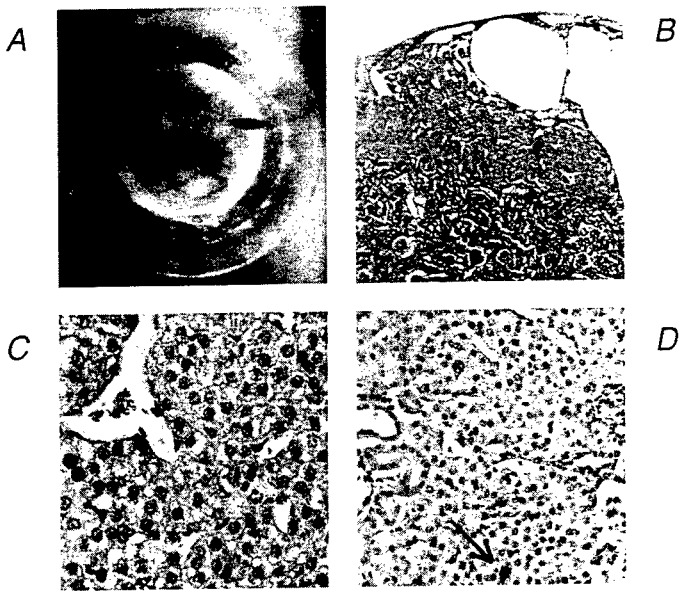

FIGURE 2

Tissue formed from transplantation of bovine adrenocortical cells observed 305 days after transplantation. ***A*** is a surface view of the tissue *in situ* within the polycarbonate cylinder, showing blood vessels entering the tissue. ***B*** and ***C*** are low- and high-power views of sections stained with hematoxylin and eosin. ***B*** shows the mouse kidney beneath the transplant tissue. ***D*** has been stained with an antibody against the proliferation marker Ki-67; one cell, indicated, is in cell division.

adrenocortical cells were followed for much longer periods. Figure 2 shows a transplant from an animal that was killed 305 days after transplantation. This female animal had 350 nM cortisol, 650 pM aldosterone and 65 nM corticosterone in the plasma at the time of sacrifice. Control nonadrenalectomized mice have 5 nM aldosterone and 700 nM corticosterone. During the 305-day period the mouse appeared healthy, maintained a normal body weight, and gave birth to four normal litters of pups. No remaining adrenocortical tissue or accessory adrenocortical nodules were found after death. The transplant tissue showed a feature not observed in any "younger" transplants, namely a large cyst within the polycarbonate cylinder, but otherwise it comprised healthy adrenocortical cells with a very low rate of cell division (Figure 2). Thus we conclude that adrenocortical xenotransplants function well over long periods in the host animal.

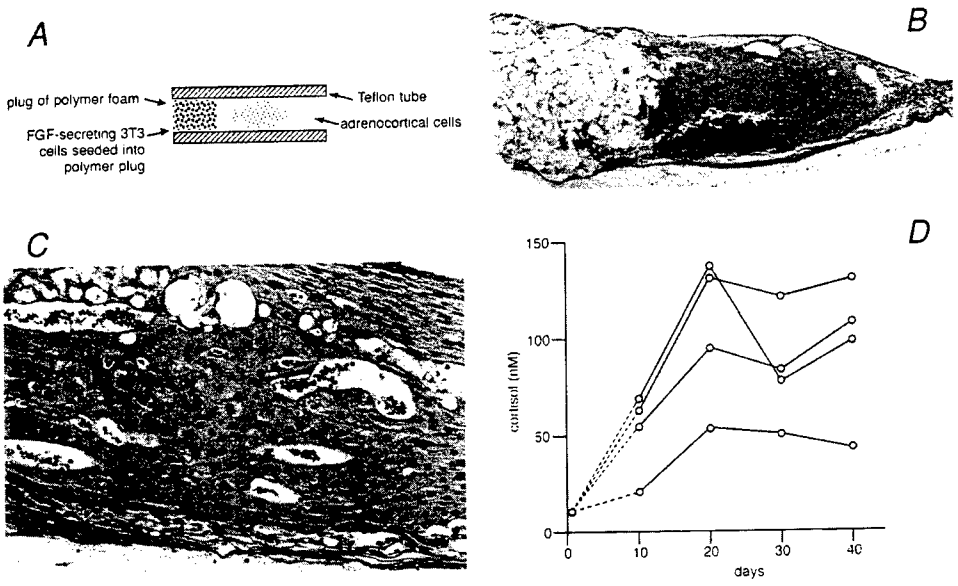

FIGURE 3

Alternate methods of cell transplantation. ***A*** shows diagrammatically a device used for bovine adrenocortical cell transplantation. The polymer foam was prepared as described in ref. 19. ***B*** and ***C*** are low- and high-power views of sections of the tissue formed, stained with hematoxylin and eosin. ***D*** shows cortisol levels of four mice bearing bovine adrenocortical cell transplants in polycarbonate cylinders attached to the outside of the kidney (see text).

Some variations of the kidney transplantation method were investigated (Table I). The polycarbonate cylinder may be attached to the outside of the kidney; the capsule was punctured to unite the space in the cylinder with the extracellular space of the kidney, and the top of the cylinder was closed with adjacent muscle tissue. The cylinder may also be made of polylactide-polyglycolide polymer or other materials. In another method, the cells were enclosed in a Teflon tube/polylactide-polyglycolide polymer device as illustrated in Figure 3. These alternate methods have been partially successful, as indicated by the histology of the tissue formed and by plasma cortisol levels (Figure 3); however, these methods do not match the success rate using subrenal capsule transplantation.

Use of Polymers for Subcutaneous Transplantation of Adrenocortical Cells: We returned to the possibility of transplanting cells subcutaneously rather than in the kidney. Several methods have been developed with varying success, as listed in

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HUMAN ADRENAL ANDROGENS. REGULATION OF BIOSYNTHESIS AND ROLE IN ESTROGEN-RESPONSIVE BREAST CANCER IN A MOUSE MODEL

Michael Thomas, Nikolay Popnikolov, Weiye Wang, S. Robert Northrup, Shiuan Chen*, and Peter J. Hornsby

Huffington Center on Aging and Dept. of Cell Biology, Baylor College of Medicine, Houston, Texas; and *Beckman Research Institute, City of Hope, Duarte, Calif.

Introduction

Human breast cancer incidence and growth are strongly influenced by hormonal steroids, estrogens and androgens. In humans, uniquely compared to other species, estrogens and androgens that affect mammary tissue originate from the adrenal gland as well as the ovaries. The major human adrenal androgen is dehydroepiandrosterone (DHEA) which is usually present in plasma as the sulfated form (DHEAS). DHEAS levels greatly exceed those of any other steroid and of most other hormonally active substances. Despite the unique aspects of adrenal physiology in humans, the function and regulation of adrenal androgen production is poorly understood. Previously, we showed that the zona reticularis is the source of all DHEA(S) in the human adrenal gland; the zona fasciculata synthesizes only the glucocorticoid, cortisol. We showed that the molecular basis for DHEA(S) biosynthesis by reticularis cells is their low expression of 3β-hydroxysteroid dehydrogenase (3β-HSD). In the work reported here, we aimed to answer basic questions about the regulation of adrenal androgen biosynthesis using the techniques of cell and molecular biology, the development of an animal model for DHEA biosynthesis and function using cell transplantation, and transplantable androgen-dependent human breast cancer in immunodeficient mice.

Experimental Procedures

Assay for DNA-binding factors: Gel mobility shift assays were performed with ^{32}P-end labeled double-stranded oligonucleotides and human adrenal whole cell or nuclear extracts.

Cell transplantation: Human adrenal cells were transplanted beneath the kidney capsule of *scid* (severe combined immunodeficiency) mice. Plasma cortisol was radioimmunoassayed.

Human breast cancer (MCF-7) cells transfected with aromatase were grown initially in *scid* mice from cultured cells and subsequently as serially transplanted tumors.

Results

Molecular basis for adrenal androgen production by the human adrenal cortex: We used probes based on a region of intron 1 of the type I 3β-HSD gene (the form expressed in non-steroidogenic tissues), which was previously shown to bind nuclear proteins, and of the type II gene, which differs significantly in sequence in this region from type I and is the form expressed in the adrenal cortex. We found at least one protein, presumably a transcription

Keywords: Adrenal androgens, MCF-7, Cell transplantation, *scid* mice, 3β-Hydroxysteroid dehydrogenase

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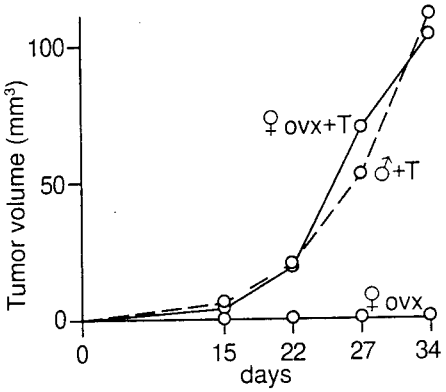
factor, present in fasciculata cells but not in reticularis cells, that binds to these probes. To characterize this factor we are using Southwestern blotting and a strategy designed to isolate the gene encoding the protein.

Human adrenal cell transplantation in *scid* mice: We found that human adrenal cells survive and function when transplanted into *scid* mice and that they replace the function of the animals' adrenal glands. When we used cells from the separated zones of the adrenal cortex, we found that transplantation of both cortisol-secreting fasciculata cells and DHEA-secreting reticularis cells resulted in approximately the same level of plasma cortisol, with low DHEAS. Thus, there appears to be a re-zonation of the cells after tranplantation. The elucidation of the putative type II 3β-HSD transcription factor will greatly aid in determining molecular mechanisms of zonation.

An alternative approach is to engineer bovine adrenal cells to make them secrete DHEA. The bovine adrenal cortex, as in almost all nonhuman species, does not make much DHEA(S), but will do so when 3β-HSD is inhibited. We are using antisense to suppress 3β-HSD in bovine cells. Although such an approach would also in principle be possible using human adrenal cells, we have found that clonal human adrenal cells cannot yet be successfully transplanted, whereas this is possible using clonal bovine adrenocortical cells. The ability to use a clone of cells means that one can genetically engineer the cells prior to transplantation. We are currently transfecting bovine cells with antisense against 3β-HSD and will be testing the ability of these cells after transplantation to provide an an animal model of high DHEA.

Growth of androgen-dependent MCF-7 tumors in *scid* mice: To test the effect of human adrenal androgens on growth of estrogen-responsive breast cancer, we have developed growth of human breast cancer in *scid* mice in an androgen-dependent manner. MCF-7 cells, a well-studied human breast cancer cell line, were transfected with aromatase cDNA, thus mimicking the normal situation of the human breast, which is exposed to estrogens derived from circulating androgens via stromal tissue aromatase. The figure shows that these cells can grow as an androgen-dependent transplantable tumor line. Future studies will be designed to assess the effect of androgens secreted by transplanted adrenal cells on tumor growth.



Growth of aromatase-transfected MCF-7 tumors in *scid* mice (ovariectomized females, and males; controls or injected wth testosterone 3x at 0, 7, 14 days, 1 mg/g) (averages of 8 mice each group)

Conclusions

Based on our previous work on the basic molecular and cellular mechanisms for the secretion of androgens by the human adrenal cortex, we have made progress in determining the transcription factors that might regulate 3β-HSD, the key enzyme characterizing the adrenal androgen-secreting zone of the human adrenal cortex. The feasibility of the use of the *scid* mouse as a model for human adrenal physiology has been demonstrated. The future determination of the mechanisms of zonation, and thereby of the formation of the adrenal androgen-biosynthesizing zone, should provide the information needed to regulate the production of the adrenal androgens, with possible therapeutic implications for the future. We have also shown that the MCF-7 breast cancer cell line can be developed as an androgen-dependent transplantable tumor, and we will be testing the growth of this tumor under the influence of the adrenal androgens.

Adrenocortical tissue formed by transplantation of normal clones of bovine adrenocortical cells in *scid* mice replaces the essential functions of the animals' adrenal glands

MICHAEL THOMAS, S. ROBERT NORTHRUP & PETER J. HORNSBY

Huffington Center on Aging and Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, USA

Xenotransplanted adrenocortical tissue of clonal origin was formed in immunodeficient (*scid*) mice by using techniques of cell transplantation. The experiments reported here used a single clone of bovine adrenocortical cells, but 5 of 20 other randomly selected clones also formed tissue. Most adrenalectomized animals bearing transplanted cells survived indefinitely, demonstrating that the cells restored the animals' capacity to survive in the absence of sodium supplementation. Formation of well-vascularized tissue at the site of transplantation was associated with stable levels of cortisol in the blood, replacing the mouse glucocorticoid (corticosterone). Ultrastructurally, the cultured cells before transplantation had characteristics of rapidly growing cells, but tissue formed *in vivo* showed features associated with active steroidogenesis. These experiments show that an endocrine tissue can be derived from a single, normal somatic cell.

Cell transplantation, an emerging field that combines elements of classical transplantation biology, *ex vivo* gene therapy and tissue engineering, has the potential to address many fundamental questions of tissue biology. It combines the power of *in vitro* techniques, such as the growth of specialized cells in culture and genetic manipulation of cultured cells, with growth of cells in an appropriate host *in vivo* to form a functional tissue[1–8]. The ability to construct endocrine tissues and organs from component cells would enable questions of cell origin, cell interactions, cell turnover, and cellular senescence within tissues to be addressed. Moreover, the application of these techniques to human cells, using a suitable host animal, would enable the resolution of questions that are unethical and impractical to perform in human subjects. To demonstrate the feasibility of cell transplantation as a technique in endocrine cell biology, in a system suitable for future application to human cells, we have developed a model in which clonal bovine adrenocortical cells are grown in culture and then transplanted into mice with the *scid* (severe combined immunodeficiency) mutation, in which they form functional adrenocortical tissue. The growth and function of bovine adrenocortical cells in culture closely resemble those of human adrenocortical cells, but their requirements for growth are less stringent, and they have a greater proliferative potential in culture[9–11]. We show that the tissue formed from clonal bovine adrenocortical cells in adrenalectomized *scid* mice is capable of replacing the essential functions of the animals' own adrenal glands. Thus, these experiments demonstrate for the first time that an endocrine tissue, replacing the host animal's organ, can be derived from a single, normal somatic cell.

Formation of tissue from clonal adrenocortical cells

A previously described clone of adrenocortical cells (no. 14 of a series of clones derived from primary bovine adrenal cortex cultures[12–15]) was expanded in culture to provide cells for transplantation. In preliminary experiments, we demonstrated that primary bovine adrenocortical cells injected beneath the kidney capsule can survive, become vascularized, and are functional. However, it was difficult to confine the cells to a single position; typically, multiple nodular groups of cells were produced by this transplantation procedure. Therefore, to confine the cells within a defined space so that the growth, vascularization and function of the cells could be more readily studied, we used a small polycarbonate cylinder to create a virtual space beneath the capsule into which the cells could be introduced, as described in the Methods section. Immediately after the cells were injected into *scid* mice, they formed a white mass almost filling the available space (Fig. 1*a*). Adrenalectomy was performed at the time of cell transplantation. When animals were killed 36 to 41 days later, pale yellow tissue was visible within the cylinders, which had been invaded by prominent blood vessels from the capsule and surrounding connective tissue (Fig. 1, *b–d*).

The cylinder was carefully removed, leaving the enclosed tissue formed from the transplanted cells intact and attached to the kidney. In some experiments, the tissue was incubated for assessment of steroidogenesis; in most experiments, it was fixed and processed for histological examination.

Most of the data presented here (Figs. 1 to 6) are from a set of 16 animals into which these clonal cells were transplanted. Similar results were obtained in other experiments using this clone; data obtained from using other clones are described later.

Survival associated with tissue formation by transplanted cells

Adrenalectomy, without sodium supplementation, is expected to be lethal[16], and this was confirmed in the strain of mice used in these experiments. Animals died 4–7 days after adrenalectomy (Fig. 2). Steroid hormone replacement for 7 days, as described in the Methods, allowed the animals to survive longer after adrenalectomy. However, when steroid administration was stopped, animals died within 5–8 days. Of 16 mice that received clonal

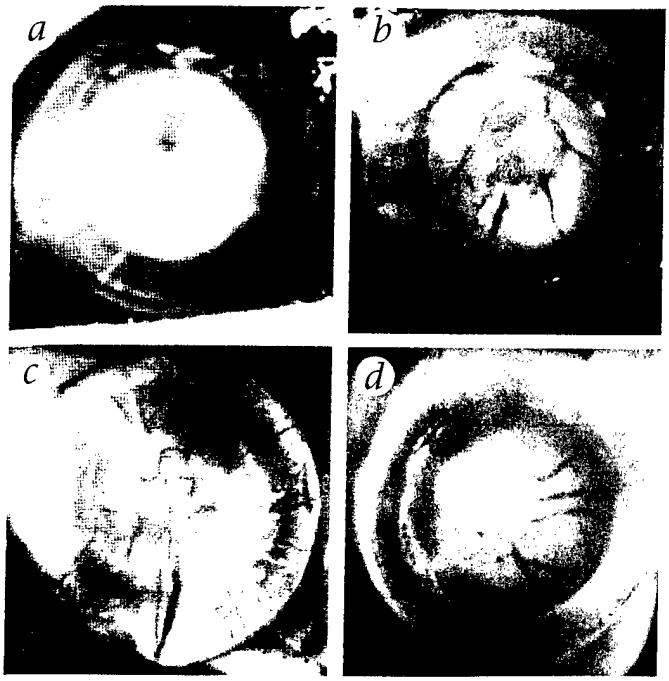


Fig. 1 Macroscopic appearance of tissue formed from clonal bovine adrenocortical cells transplanted into polycarbonate cylinders beneath the kidney capsules of *scid* mice. *a*, cells immediately after transrenal injection. *b*, *c* and *d*, Tissue formed from the clonal cells in three animals 36–41 days after transplantation. Original magnification, ×10.

adrenocortical cells, 15 survived through the period during which death of the sham-operated animals occurred, demonstrating that the transplanted cells restored the animals' capacity to survive in the absence of sodium supplementation. Two animals died at a later time, associated with only partial development of vascularized tissue of the type illustrated in Fig. 1. Overall, in this representative experiment, 81% of animals that received clonal bovine adrenocortical cells survived indefinitely and developed functional vascularized tissue from the transplanted cells.

Time course of cortisol and corticosterone levels in plasma

Animals bearing transplanted clonal adrenocortical cells and surviving beyond 14 days had the bovine glucocorticoid, cortisol, in their blood. Cortisol is not produced by the mouse adrenal cortex because the mouse adrenal does not express the steroid 17α-hydroxylase, resulting both in the production of corticosterone rather than cortisol as the major glucocorticoid and also in the lack of adrenal androgen synthesis, which requires this enzyme[17–19].

Corticosterone levels fell to essentially zero immediately after adrenalectomy and cell transplantation; they remained low, but increased gradually (Fig. 3*a*). Cortisol rose from a measured pretransplantation value of ~15 nM to a peak level of ~75 nM at 30 days after cell transplantation. The pretransplantation cortisol value probably represents cross-reaction of corticosterone in the radioimmunoassay, rather than authentic cortisol (see Methods). However, the strong decline in plasma corticosterone after adrenalectomy and cell transplantation shows that such cross-reaction has a negligible impact on cortisol measurements in the experimental animals.

As described in the Methods section, in most experiments clonal bovine adrenocortical cells were transplanted with fibroblast growth factor (FGF)-secreting 3T3 cells; when these cells were omitted, the formation of functional tissue was impaired. The tissue formed was smaller and not as well vascularized. Two such animals are shown as C in Fig. 3*a*. In all other experiments reported here, FGF-secreting 3T3 cells were included with the adrenocortical cells.

The data of Fig. 3*a* show that the mouse glucocorticoid, corticosterone, was replaced with the bovine glucocorticoid, cortisol. To confirm that cortisol was produced only by tissue formed from the transplanted cells, the left kidney bearing the adrenocortical tissue was removed and the animals allowed to survive a further 3 days, a period predicted to be less than the time to death resulting from deprivation of adrenal steroids (based on the data in Fig. 2). In these animals plasma cortisol was essentially zero at the time they were killed (Fig. 3*b*).

Structure of tissue formed from clonal adrenocortical cells

Tissue formed from transplanted clonal adrenocortical cells was fixed and processed for conventional histology and histochemistry. In the animals that had high plasma cortisol and well-developed vascularization, the tissue formed from the transplanted cells had a relatively uniform structure (Fig. 4). The tissue was bounded by the cylinder (removed before histological processing) on the lateral surfaces, the capsule on the upper surface and the kidney parenchyma on the lower surface. Staining with hematoxylin and eosin showed the tissue to have an appearance generally similar to that of the normal adrenal cortex. Fig. 4*d* shows cords of cells and sinusoidal capillaries resembling those of the zona fasciculata of the normal cortex[20] (Fig. 4*e*).

Ultrastructural features of transplant tissue

In general, the ultrastructure of tissue formed from transplanted clonal cells closely resembled that of the normal adrenal cortex and differed greatly from that of the cells in culture before trans-

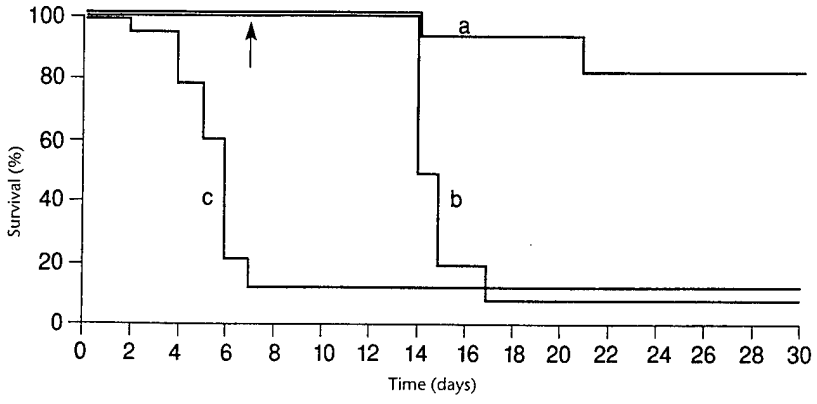


Fig. 2 Survival of mice after adrenalectomy and transplantation of clonal adrenocortical cells. The three groups of animals are as follows: a, adrenalectomy plus cell transplantation at day 0, followed by 7 days dexamethasone and fludrocortisone (16 animals); b, adrenalectomy on day 0, 7 days dexamethasone plus fludrocortisone (10 animals); c, adrenalectomy only on day 0 (23 animals). The termination of steroid administration in groups a and b is indicated by the arrow.

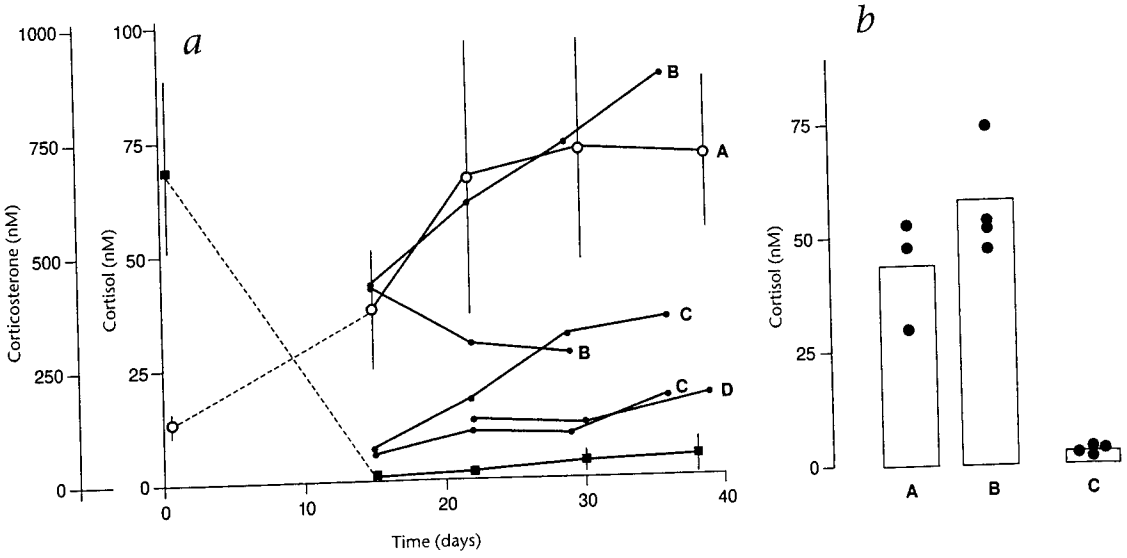


Fig. 3 *a*, Time course of changes in plasma cortisol and corticosterone in 16 animals with transplanted clonal adrenocortical cells. The abscissa indicates the time in days since cell transplantation. *a*, Average values (±s.d.) for 10 animals that received 2 × 10^6 clonal adrenocortical cells with 4 × 10^5 3T3 cells. The two individual curves marked B are data from two additional animals that received 4 × 10^6 clonal adrenal cells and 8 × 10^5 3T3 cells. The two curves marked C are data from two animals that received 2 × 10^6 bovine adrenal cells without 3T3 cells. D shows one animal, otherwise the same as A, that had high corticosterone with low cortisol and is presumed to have developed accessory adrenocortical tissue after adrenalectomy[41]. Squares show average values (±s.d.) for corticosterone in the animals in groups A, B, and C. *b*, Change in plasma cortisol after removal of the kidney with transplanted clonal cells in an additional four animals 30–40 days after transplantation. Values and averages for plasma cortisol are shown for blood samples taken approximately 2 weeks (A) and 1 week (B) before transplant removal and at the time animals were killed, 3 days after nephrectomy (C).

plantation. Mitochondria in transplanted cells often had cristae in the tubular or vesicular forms characteristic of steroidogenic tissues[21], and also demonstrated here in normal bovine adrenal cortex (Fig. 5). In contrast, mitochondria in pretransplantation cells in culture showed only lamellar cristae. Whereas pretransplantation cells had large numbers of ribosomes and prominent rough ER, transplanted cells showed extensive development of smooth ER, less rough ER and fewer free ribosomes, features also characteristic of steroidogenic tissue[21] and observed here in the intact adrenal gland. Another prominent feature of the tissue formed from the transplanted cells was extensive contacts between cells consisting of many interdigitating microvilli, which were also observed in the subendothelial space. These have been described previously in the mouse and rat adrenal cortex[22–24] and were observed here in the bovine gland, but not in the cells in culture (Fig. 5).

The ultrastructure of the capillaries also resembled that of the normal adrenal cortex. Fenestrae were present in the mouse endothelial cells that have invaded the tissue derived from the transplanted cells; fenestrated endothelia are typical of endocrine tissues, and in the adrenal cortex increase in number in response to stimulation by adrenocorticotropic hormone (ACTH)[25,26].

Proliferation of adrenocortical cells

Thirty-six to 41 days after transplantation, some cells continue to proliferate, as evidenced by expression of the Ki-67 nuclear antigen. Although the fraction of proliferating cells was variable, it was comparable to that of the young adrenal cortex *in vivo* (Fig. 6). Pretransplantation cells in culture had a much higher rate of proliferation, consistent with their essentially exponential growth at this stage of their culture life span (see the Methods).

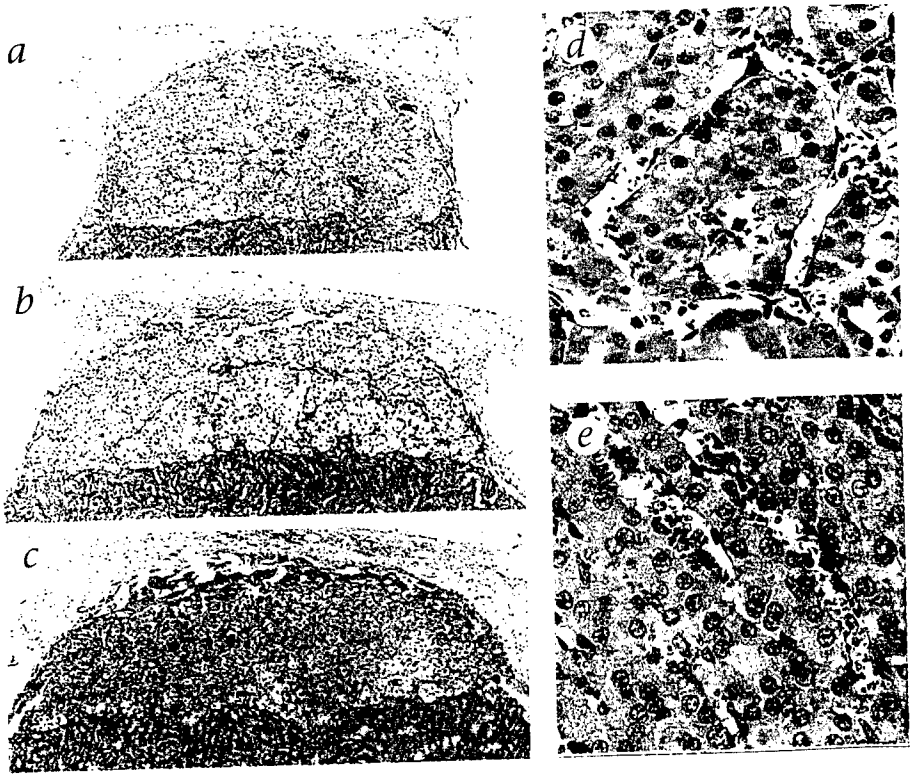


Fig. 4 Histological appearance of tissue formed from transplanted clonal adrenocortical cells. *a*, *b* and *c*, Representative samples of the tissue formed from the cells, hematoxylin/eosin stain. Original magnification, ×80. *d* and *e*, Structure of tissue in comparison with normal adrenal cortex. *d*, Tissue formed from clonal bovine adrenocortical cells; the junction with the mouse kidney is visible at the bottom. *e*, Normal bovine adrenal cortex. Original magnification, ×400.

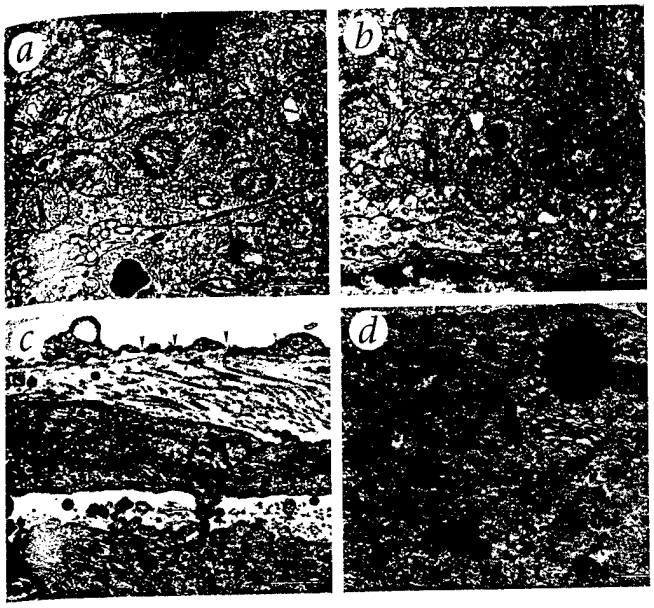


Fig. 5 Ultrastructure of tissue formed from clonal adrenocortical cells. *a* and *c*, Tissue formed from transplanted clonal adrenocortical cells; *b*, normal bovine adrenal cortex; *d*, clonal cells in culture. Large arrows indicate microvilli. Small arrows show fenestrae in the endothelium formed within the adrenocortical tissue. Scale bars, 500 nm.

Tissue formation by transplantation of other adrenocortical clones

To test whether the clone (no. 14) used in these experiments was uniquely able to form adrenocortical tissue when transplanted, 20 new clones were derived from primary bovine adrenocortical cells, as described in the Methods, and were transplanted into *scid* mice. Of these, five formed tissue similar in structure to that produced by clone no. 14, rescued the animals from the lethal effects of adrenalectomy, and produced plasma cortisol levels >50 nM. Animals with transplanted cells from the other clones died after cessation of the 7-day steroid replacement; plasma cortisol was <20 nM, and no adrenocortical tissue was observed at the site of transplantation.

Discussion

Formation of tissue in *scid* mice

The formation of functional adrenocortical tissue from clonal bovine adrenocortical cells grown in culture demonstrates that endocrine tissue, functionally replacing the corresponding tissue of the animals, can be derived from a single, normal somatic cell. Tissue formed from the transplanted cells replaced the essential functions of the animals' own adrenal glands because the animals survived adrenalectomy and the bovine glucocorticoid, cortisol, replaced the mouse glucocorticoid, corticosterone. Cortisol is equipotent with corticosterone in occupying the mouse glucocorticoid receptor[27]. In some cases, we observed that the transplanted cells also secrete aldosterone (unpublished data). However, we did not determine which steroids produced by the cells replace the mineralocorticoids normally secreted by the adrenal glands, loss of which is presumably the cause of death in adrenalectomized animals without transplanted cells[16].

There was no evidence of an immune reaction to the transplanted cells, consistent with the absence of B and T cell-mediated immunity in the *scid* mouse[28]. The tissue was a chimeric structure composed of bovine and mouse cells; the cells appeared healthy and there was no obvious evidence of cell death. There was abundant vascularization, supported by the cotransplanted FGF-secreting 3T3 cells, some of which appear to become integrated into the capillary endothelium (unpublished observations). Forty days after transplantation there was continued proliferation of the transplanted cells, indicating that the cells are capable of long-term proliferation in the host animal.

Transplantation of endocrine cells

It is unknown to what extent the current findings may apply only to the adrenal cortex or might be generally applicable to endocrine tissues. For example, transplanted human thyroid tissue survives and functions in *scid* mice, and human thyroid cells grown as a nonclonal mass population in culture can be transplanted[29,30]. However, xenotransplanted tissue or cells were not demonstrated to replace the animals' thyroid function. Many tissues in the endocrine system show the ability to regenerate from small tissue remnants, suggesting that there is a large potential for individual differentiated cells or stem cells to repopulate and reconstitute a functional organ[31,32]. It is not known to what extent this ability depends on the properties of the endocrine parenchymal cells and to what extent it depends on the contributions of the vascular system, or stromal or other nonparenchymal elements. *In vivo* experiments on regeneration do not allow unambiguous conclusions about the relative contributions of different cell types, but cell transplantation offers the ability to answer such questions.

Fig. 6 Cell proliferation in tissue formed from clonal bovine adrenocortical cells. *a* and *b*, Tissue from two animals showing expression of the Ki-67 proliferation-associated antigen. *c*, Normal adrenal cortex from a young animal. *d*, Clonal adrenocortical cells in culture (suspended into a thrombin clot for sectioning, as described in the Methods section). Original magnification, ×100.

The nature of clonable adrenocortical cells

Although most of the experiments described here used one particular bovine adrenocortical cell clone, we also demonstrated that the ability to form functional tissue *in vivo* is not confined to a single clone. After transplantation, 25% of unselected newly derived clones were able to form tissue. Clones have not undergone genetic modification before transplantation and have a finite proliferative culture life span[12-15]. The phenotype of the adrenocortical cells that generated these clones cannot be directly ascertained. Although it is possible that the originating cells might be a form of stem cell or some other subpopulation with properties not shared by most cells of the adrenal cortex, the available evidence suggests that they are typical differentiated adrenocortical cells. Almost all cells in primary bovine adrenocortical cell cultures express differentiated features and yet are also capable of DNA synthesis[10]. The kinetics of cell proliferation in the adrenal cortex *in vivo* are compatible with an extensive capacity for self-renewal of most cells in the tissue[33]. Future experiments should be able to resolve these issues.

Adaptation of the transplanted cells to the host animal

Previously, we showed that the clone used here and other similar bovine adrenocortical cell clones, at a time in culture when first expanded sufficiently for study, do not synthesize cortisol when incubated with cyclic AMP and insulin-like growth factor I (IGF-I), both of which are required for induction of the steroidogenic enzyme genes[15]. Because of low expression of the later part of the pathway, 21-hydroxylase and 11β-hydroxylase, cells synthesize deoxycortisol and 17α-hydroxyprogesterone but not cortisol. When the cells were embedded in extracellular matrix (Matrigel), however, stimulation with cyclic AMP and IGF-I resulted in the induction of 21-hydroxylase and 11β-hydroxylase and synthesis of cortisol[15]. The factors involved were not identified. However, the cells in Matrigel adopted a cord-like structure with possibly different cell–cell contacts[15]. The fact that the same cells were capable of cortisol synthesis when transplanted *in vivo* suggests that the three-dimensional structure formed *in vivo* and the structures formed by the cells cultured in Matrigel share some common feature enabling appropriate expression of differentiated function genes[34,35].

The bovine adrenocortical tissue formed from the transplanted cells was clearly capable of proper function in the mouse. Mouse ACTH, as expected from the near-identity of mouse and bovine ACTH (ref. 36, 37) was evidently able to activate plasma membrane receptors on bovine adrenocortical cells. The tissue was also apparently able to use mouse lipoproteins for steroidogenesis; the relative usage of high-density and low-density lipoproteins for steroidogenesis among rodent and nonrodent species is not yet defined[24].

The ability to create xenotransplanted clonal endocrine tissues in host animals offers the possibility for future genetic manipulation of the cells before transplantation, which would enable the resolution of multiple questions in endocrine physiology, cell biology and molecular biology. Clones such as that used here, with extensive remaining proliferative potential, could be used to form transplanted tissue in an essentially unlimited number of animals. Moreover, the formation of a mouse now reconstituted with a xenotransplanted cell type enables the physiology of such animals to be investigated and to perform experiments that would be impractical or impossible in the donor species.

Methods

Growth of bovine adrenocortical cells in culture. The principal clone (no. 14) of bovine adrenocortical cells used in these experiments was derived and described previously[12-15]. The derivation of the clone from a primary culture of bovine adrenocortical cells was similar to that described here for newly prepared clones. Cells were frozen in liquid nitrogen shortly after the initial expansion of the clone to ~4×10^6 cells, at which stage they are at approximately one-third of their total proliferative potential ("life span") in culture[12]. Frozen cells were thawed and replated in Dulbecco's Eagle's medium/Ham's F-12 1:1 with 10% fetal bovine serum, 10% horse serum and 0.1 ng/ml recombinant basic FGF (Mallinckrodt, St. Louis, MO). This was supplemented with 1% (vol/vol) UltroSer G (Biosepra, Villeneuve-la-Garenne, France), a mixture of growth factors that improve clonal adrenocortical cell growth[9,11].

For the preparation of new clones, primary bovine adrenocortical cells, dissociated by enzymatic and mechanical dispersion of adrenocortical tissue[9,11], were plated at limiting dilution in 10-cm plates in the same medium. Visual inspection of the plated cells verified the absence of pairs or groups of cells. When small colonies had formed, they were mechanically isolated using a pipette tip and were transferred sequentially to larger dishes by subculture with bacterial protease[9,11].

FGF-transfected 3T3 cells. A line of 3T3 cells stably expressing FGF-1 fused in frame with a signal peptide from hst/KS3, yielding a highly angiogenic secreted product[38], was generously supplied by T. Maciag. 3T3 cells were grown under the same conditions as bovine adrenocortical cells. At ~20% confluence, cells were incubated for 24 h with 2 μg/ml mitomycin C (ref. 39; Sigma Chemical Co.). Mitomycin C treatment rendered the entire population incapable of further division, as demonstrated by the lack of colony growth in cells subcultured to low density.

Transplantation of cells beneath the kidney capsule of *scid* mice. ICR *scid* mice originally purchased from Taconic (Germantown, NY) were maintained in an animal barrier facility as a breeding colony. Animals (both males and females) at an age greater than 6 weeks (~20 g body weight) were used in these experiments. Under tribromoethanol anesthesia, mice were adrenalectomized and were transplanted with adrenocortical cells in a single procedure. The kidney on the left side was exteriorized and a small transverse incision was made through the capsule near the inferior pole. Using one point of a fine forceps, a pocket was created under the capsule. To provide a chamber for the transplanted cells a 1-mm length of polycarbonate tube (3-mm internal diameter) was surface-polished by brief exposure to dichloromethane vapor. This cylinder was pushed partially into the pocket under the capsule, filled with culture medium, and then introduced fully into the pocket so that the capsule on the top and the kidney parenchyma on the bottom formed a sealed space.

Adrenocortical cells were introduced into this space as follows. Cells were released from the culture dish by digestion with bacterial protease[9,11]. In most experiments, adrenal cells were mixed in a ratio of 5:1 with mitomycin C-treated 3T3 cells, typically 2×10^6 adrenocortical cells and 4×10^5 3T3 cells. The cells were introduced into the subcapsular cylinder in a small volume of culture medium by a transrenal injection using a 50-μl Hamilton syringe fitted with a blunt 22-gauge needle. Post-operative care for the animals consisted of the administration in the drinking water of a mixture of analgesics and antibiotics, but additional sodium, beyond that already supplied in the normal diet, was not given via the drinking water.

For 7 days after surgery, animals were injected subcutaneously once daily with 1 μg/g body weight fludrocortisone acetate and dexamethasone phosphate[40]. Blood samples for assessment of circulating adrenal steroid levels were not taken during this period because preliminary tests showed that adrenal steroidogenesis was suppressed by administration of the steroids. One week after cessation of the steroid administration, and at approximately weekly intervals thereafter, blood samples were taken 15 minutes after the injection of ACTH (Sigma, 0.01 units per gram body weight). Animals were killed for analysis of histology 30–50 days after cell transplantation.

Histology. The fixation, paraffin embedding and histological examination of tissue formed from transplanted cells was carried out using standard techniques. For comparison with tissues, cultured cells were removed from the culture dish by proteolytic digestion and were embedded in a thrombin/fibrin clot. The clot was fixed and processed in the same way as the tissues.

Sections were stained for expression of the Ki-67 proliferation-associated antigen using monoclonal antibody MIB-1 (Immunotech, Westbrook, ME) and avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA), as recommended by the manufacturers. Sections were lightly counterstained with hematoxylin. This antibody does not recognize the mouse homologue of Ki-67 but recognizes the bovine protein with staining equivalent to that observed with human tissues (unpublished observations).

Electron microscopy. Tissue formed from transplanted cells, control adrenal glands, and cultured adrenocortical cells were fixed with glutaraldehyde and osmium tetroxide and embedded in Epon. Cultured cells were removed from the culture dish by scraping after fixation and before embedding. Sections (100 nm thick) were stained with uranyl acetate and lead citrate and were examined and photographed by electron microscopy.

Radioimmunoassays. Radioimmunoassays were performed using the following kits. Cortisol: Corti-cote (ICN Pharmaceuticals, Orangeburg, NY). The manufacturer reports that this antibody has a cross-reaction of 1.2% with corticosterone. Corticosterone: ImmuChem double-antibody kit (ICN). This antibody has a reported cross-reaction of 0.05% with cortisol. Radioimmunoassays were performed by direct assays on appropriate volumes of serum from the animals. Measured values of cortisol in intact (nonadrenalectomized) animals probably do not represent authentic cortisol because the cross-reaction of the cortisol antibody with corticosterone would give the values observed.

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1 The Biosynthesis of DHEA by the Adrenal Cortex and its Age-related Decline

Peter J. Hornsby

Huffington Center on Aging, Baylor College of Medicine, 1 Baylor Plaza M320, Houston, Texas 77030 USA

Abstract

Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) are secreted in high amounts by zona reticularis (ZR) cells of the adrenal cortex in adult humans. The concept emphasized in this chapter is that the ZR cell of the human adrenal cortex is a unique cell type with the specific function of secreting DHEA(S), although to date the biology of the ZR cell has received little attention. The key feature of the ZR cell is its low expression of 3β-hydroxysteroid dehydrogenase, which prevents the conversion of steroid precursors to cortisol and permits the biosynthesis of DHEA. ZR cells do not appear in the adrenal cortex until adrenarche, the time in late childhood when these cells differentiate in sufficient numbers to cause a substantial rise in plasma levels of DHEAS. Adrenarche is observed only in species closely related to humans, and not in most primates or other species. There is a steep decline in DHEA(S) biosynthesis in aging, most likely resulting from a decrease in the number of functional ZR cells. Differences in gene expression between ZR cells and other adrenocortical cells may cause ZR cells to be primed to undergo apoptosis and/or to be eliminated by the immune system. Cell loss may be the result of normal cell turnover or may occur during occasional periods of ischemia or other injury to the gland.

The appearance of DHEAS in plasma in late childhood and its disappearance with age presents a puzzle that lacks satisfactory biological explanations, although the concept that the age-related decline results from death and non-replacement of the cells of the zona reticularis forms a working hypothesis on which to base future studies.

KEYWORDS: Adrenal cortex, zona reticularis, 3β-hydroxysteroid dehydrogenase, adrenarche, aging, apoptosis

THE FUNCTION OF THE ZONA RETICULARIS OF THE ADRENAL CORTEX: THE SECRETION OF DHEA(S)

In humans and a few other primates, dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) (collectively designated DHEA(S)) are secreted in high amounts by the adrenal cortex. In adult humans, DHEAS circulates at very high concentrations (~10 μM), greatly exceeding the levels of other steroid hormones (Parker and Odell, 1980; Guillemette *et al.*, 1996). Plasma DHEAS levels reflect synthesis by the adrenal cortex because the adrenal is normally the source of almost all DHEA(S) (Parker and Odell, 1980). Small amounts of

DHEA are also synthesized by the gonads. Because unconjugated DHEA is essentially an intermediate (to and from DHEAS or other steroids), plasma levels of this steroid are not considered to be very significant (Baulieu, 1996), in contrast to DHEAS, which is slower to change because of its very low metabolic clearance rate (Kellie and Smith, 1957; Wang *et al.*, 1967; Haning *et al.*, 1989).

Adrenal production of DHEA(S) is negligible or absent in most laboratory and domestic animals, including rats, mice, guinea pigs, dogs, pigs, and cattle (Guillemette *et al.*, 1996). The adrenal glands in the adult mouse and rat lack the 17α-hydroxylase enzyme needed for the biosynthesis of DHEA (Perkins and Payne, 1988; van Weerden *et al.*, 1992; Keeney *et al.*, 1995). Other species express the enzyme in their adrenals but do not make significant quantities of DHEA(S), for reasons that are discussed here.

The adult adrenal cortex comprises three zones, the outer zona glomerulosa (ZG), the middle zona fasciculata (ZF), and the inner zona reticularis (ZR). The three zones of the adrenal cortex produce different steroids because of different patterns of gene expression in the three zonal cell types (see Figure 1). The concept emphasized in this chapter is that the ZR cell of the human adrenal cortex is a unique cell type with the specific function of secreting DHEA(S). The biology of the ZR cell has received little attention. This has resulted from three factors:

First, only in humans and very closely related species do ZR cells have a function and pattern of gene expression that differs from that of ZF cells. In most species, such as the rat, cow, rabbit, and dog, a morphological reticularis zone is present but its function is not distinct from that of the fasciculata. In a few species, such as the guinea pig, ZR cells differ

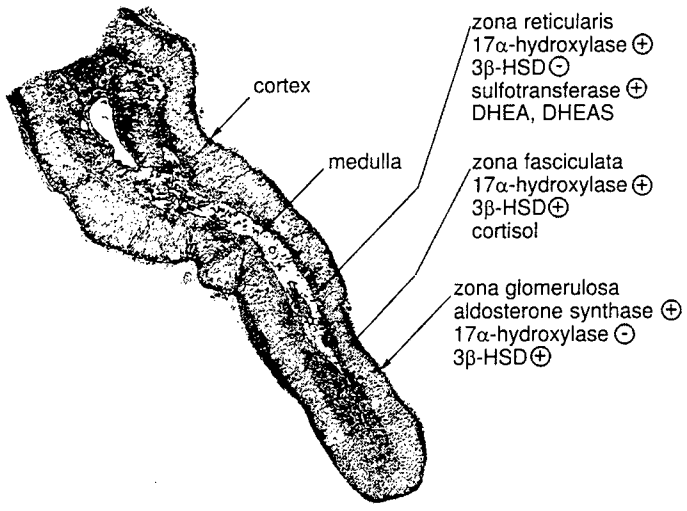

Figure 1. DHEA and DHEAS are secreted by a discrete layer of cells in the adult human adrenal cortex, the zona reticularis (ZR). The key molecular feature of ZR cells that results in the production of DHEA(S) is their low expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) (Endoh *et al.*, 1996). The zona fasciculata (ZF) has a high level of expression of type II 3β-HSD enabling the synthesis of the glucocorticoid, cortisol. Other important enzymatic differences between the zones that result in the production of different steroids are shown. (Reproduced from Hornsby, 1997; adrenal gland section reproduced from Bargmann, 1951.).

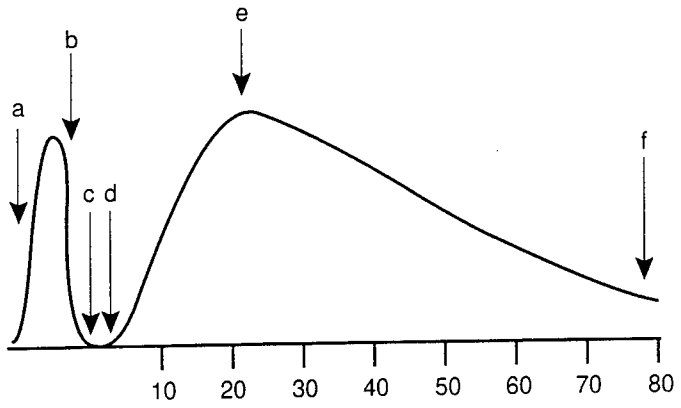

Figure 2. Diagrammatic representation of the plasma levels of DHEAS over the life span in humans. Before birth (a) the fetal zone of the adrenal cortex secretes large amounts of DHEA(S); following birth (b), the fetal zone rapidly involutes (c). The role of fetal DHEA(S) is outside the scope of this chapter (see Mesiano and Jaffe, 1992; Pepe and Albrecht, 1995). By about age 6–7, ZR cells have developed in the adrenal cortex and plasma DHEAS levels begin to rise (adrenarche) (d). The achievement of the peak level of plasma DHEAS in young adulthood (e) is followed by a progressive decline in adrenal secretion of DHEA(S), so that plasma DHEAS levels are often very low by age 70 and beyond (f) (Parker and Odell, 1980). The ordinate represents age in years; the scale is expanded before 10 years of age. (Reproduced from Hornsby, 1997)

from ZF cells in substantial ways; however, there is no evidence that ZR cells in such species produce DHEA(S), in more than trace amounts (Hyatt *et al.*, 1983a; Hyatt *et al.*, 1983b). The fact that there is no suitable animal model has resulted in little biological study of ZR cell function and a lack of appreciation that this cell has unique functions in humans.

Second, the function of its steroid products, DHEA and DHEAS, is unclear. As I have discussed elsewhere (Hornsby, 1997), these steroids are usually and correctly termed "adrenal androgens", because, although they lack direct androgen activity, they are readily converted into active androgens in many tissues, but whether this is their major function is not known. From arguments presented here, it is inescapable that these steroids have an essential function, despite our ignorance of its nature. The lack of suitable animal models has severely impeded progress in this area.

Third, most studies of ZR cells have been confined to histological techniques (Gell *et al.*, 1996; Parker, 1997). Prior to the isolation and study of ZR cells in culture in this laboratory (Endoh *et al.*, 1996), only a single experiment in the literature showed differences in ZR and ZF cell function based on a physical separation of the cell types (Hyatt *et al.*, 1983a). Study of the separated cells in culture has rapidly shown that they differ in many respects in their patterns of gene expression (P.R. Casson, W. Wang, and P.J. Hornsby, unpublished observations).

The adrenal cortex has three functional zones only in adult life, from pre-puberty to old age (see Figure 2). ZR cells do not appear in substantial numbers in the adrenal cortex until about age 6–7 (Figure 3), when plasma DHEAS begins to rise (adrenarche) (Cutler *et al.*, 1978; Rich *et al.*, 1981; Rosenfield, 1994). Before adrenarche, the cortex comprises morphologically only glomerulosa and fasciculata zones and makes no DHEA(S).

The key feature of the ZR cell is its low expression of type II 3β-hydroxysteroid dehydrogenase (3β-HSD), as shown by histochemistry (Sasano *et al.*, 1990; Gell *et al.*, 1996; Parker, 1997) and by several techniques in isolated ZR cells (Endoh *et al.*, 1996). At the mRNA level, the expression of 3β-HSD in pure ZR cells is close to zero, whereas it is much higher in ZF cells, which do not produce DHEA. The key role of 3β-HSD is most simply demonstrated by chemical inhibition of its enzymatic activity, which causes both human ZF cells and bovine adrenocortical cells, both of which normally synthesize minimal amounts of DHEA, to produce large quantities of this steroid (Hornsby, 1980; Endoh *et al.*, 1996). The biochemical basis for this phenomenon is shown in Figure 3.

The reticularis is not merely a morphological entity apparent only in histological sections, but comprises a distinct cell in the same way that the morphologically distinct glomerulosa corresponds to a cell type distinct from that of the fasciculata. Consequently, one must consider the physiology, cell biology, and molecular biology of each zonal cell type separately. For example, we would be in error if we were considering the secretion of aldosterone and cortisol by the adrenal cortex and we assumed that, within a single cell type, the activities of 17α-hydroxylase and aldosterone synthase are regulated so as to produce more cortisol or more aldosterone. Clearly this does not happen; within a single cell type, there is no diversion toward the aldosterone or cortisol pathway. Similarly, there is no diversion of steroid precursor to or away from DHEA(S) versus cortisol or aldosterone. The synthesis of these steroids takes place in separate cells, each dedicated to the

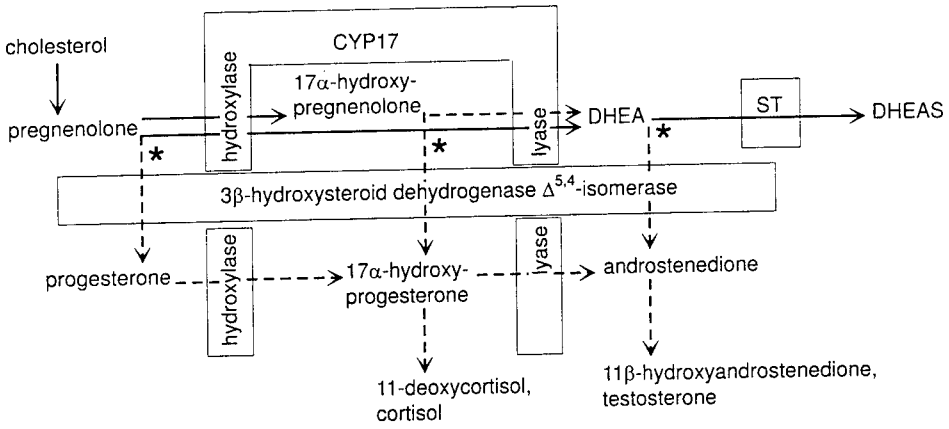

Figure 3. DHEAS biosynthesis results from limitation of 3β-HSD activity. The three asterisks indicate the three points where there is competition between 3β-HSD and another enzyme for substrate, points where the limitation of 3β-HSD activity keeps the steroid in the Δ^5 pathway. Dashed arrows indicate blocked or limited conversion pathways. The first two points of competition are between 3β-HSD and the two different enzyme activities, 17α-hydroxylase and $C_{17,20}$-lyase, of a single protein, CYP17 (Zuber *et al.*, 1986; Swart *et al.*, 1989). In ZR cells, the limitation of 3β-HSD activity diverts pregnenolone to 17α-hydroxypregnenolone, which is then acted on by the much higher lyase activity of the CYP17 protein for Δ^5 substrates, with production of DHEA. The key enzymatic differences of low 3β-HSD activity and high DHEA sulfotransferase (ST) activity combine to ensure that DHEA and DHEAS are the major products; ~50% of DHEA is transformed by DHEA sulfotransferase to DHEAS. (Reproduced from Hornsby, 1995)

formation of steroids with specific biological activity. Therefore, one must be cautious when interpreting changes in levels of circulating adrenal steroids (e.g. at adrenarche) in terms of changes in adrenal enzyme activities.

THE NATURE OF ADRENARCHE

Adrenarche is the time in childhood when ZR cells differentiate in sufficient numbers in the adrenal cortex to cause a substantial rise in plasma levels of DHEAS. Adrenarche is observed only in species closely related to humans such as the gorilla and chimpanzee (Cutler *et al.*, 1978; Smail *et al.*, 1982). Primate species outside of this group, such as the rhesus and Cynomolgus monkeys, have measurable levels of DHEAS in plasma but do not undergo adrenarche and do not maintain the high adult levels of DHEAS found in humans (Koritnik *et al.*, 1983; Meusy-Dessolle and Dang, 1985; Guillemette *et al.*, 1996; Lane *et al.*, 1997).

What factors at the time of adrenarche cause the differentiation of a new cell type within the adrenal cortex? Presumably, although this is not unequivocally established, ZR cells derive from existing ZF cells (Dhom, 1973). As the adrenal cortex grows in thickness, ZR cells may differentiate in response to signals existing on the venous end of the capillary bed; i.e., the differentiation signal may be a factor that accumulates or is depleted across the long capillary bed of the adrenal cortex (see figure in Hornsby, 1995). Although I have suggested that such a gradient could be comprised of adrenal steroids (Hornsby, 1985), this idea has no greater merit than the gradient being formed of any other substance produced or transformed by adrenocortical cells. The signal for the differentiation of ZR cells appears to be of adrenal origin, but its biosynthesis may be influenced by pituitary factors. Adrenarche does not take place in individuals with congenital unresponsiveness to ACTH (Weber *et al.*, 1997) or in individuals with congenital adrenal hyperplasia who have received long-term glucocorticoid therapy (Conway *et al.*, 1982). Some cases of precocious adrenarche are associated with growth hormone and prolactin excess (Iwatani *et al.*, 1992). The adrenal cortex has a relatively high level of prolactin receptors in several species (Posner *et al.*, 1974; Bolander *et al.*, 1976; Glasow *et al.*, 1996; Freemark *et al.*, 1997). In women, particularly patients with prolactin-secreting tumors, there is a correlation between prolactin levels and DHEAS (Yamaji *et al.*, 1989). Prolactin has a growth-promoting effect in many tissues (Sauro and Zorn, 1991; Girolomoni *et al.*, 1993). Growth hormone receptors are present in the adrenal cortex (Lin *et al.*, 1997; Zogopoulos *et al.*, 1996). Additionally, in older individuals, low DHEAS levels are increased on administration of MK0677, a drug which restores pulsatile growth hormone secretion by the pituitary (Smith *et al.*, 1996).

The biological significance of the greatly increased secretion of DHEA(S) by the adrenal cortex at adrenarche is unknown (Hornsby, 1997). One can make a strong argument, based on evolutionary considerations, that there must be a function of DHEA in human physiology (Hornsby, 1997). DHEA is produced by the adrenal glands at a critical time during the human life span, just prior to puberty, and DHEAS circulates at an extraordinarily high level in young adults. If DHEA had a neutral or harmful effect on young adult physiology, the production of DHEA by this separate cell type, the ZR cell, would have been rapidly eliminated by natural selection during evolution. However, the opposite,

in fact, occurred. Humans evolved from precursor species which produced much less DHEA. Monkeys such as the rhesus macaque and the crab-eating macaque have a low level of plasma DHEAS that is at its highest in the newborn animal, and declines continuously thereafter, particularly at puberty (Meusy-Dessolle and Dang, 1985; Lane *et al.*, 1997). Therefore, their DHEA(S) appears to originate from a persistent fetal zone in the adrenal cortex, rather than from ZR cells that arise de novo at adrenarche. The latter feature of the human life span is incompatible with either a neutral or harmful role for DHEA, but must imply a positive role for some aspect of young adult health and reproduction.

DHEAS and DHEA serve as a source of active androgens in a subset of androgen-responsive tissues (Labrie, 1991; Ebeling and Koivisto, 1994). These steroids are often referred to as adrenal androgens, although this term is also used to refer to another steroid, androstenedione, which is also secreted by the adrenal cortex under some circumstances, and which may be converted to testosterone in target tissues. However, androstenedione is secreted in large amounts only in genetic deficiency of 21-hydroxylase (White and New, 1992). 21-Hydroxylase deficiency of the form termed "simple virilizing" results in masculinization of affected females at birth, because the block of 21-hydroxylase leads to the accumulation of 17α-hydroxyprogesterone, which serves as a precursor for androstenedione (White and New, 1992) (see Figure 3). However, in normal individuals most androstenedione in androgen-sensitive tissues is formed from DHEA, via conversion by type I 3β-HSD (Labrie, 1991). The increase in adrenal androgen production at adrenarche is restricted to DHEA and DHEAS; there is no evidence that androstenedione production by the adrenal glands increases. Because androstenedione can be formed peripherally from DHEA(S), and directly by the gonads, circulating levels do not necessarily reflect adrenal production rates. A surrogate marker for adrenal production of androstenedione is 11β-hydroxyandrostenedione. The 11β-hydroxylase enzyme that is required for its formation is expressed only in the adrenal glands and so this steroid is adrenal-specific (Holownia *et al.*, 1992). It is significant, therefore, that 11β-hydroxyandrostenedione does not increase at adrenarche and, in fact, is more or less constant throughout childhood and puberty (Lejeune-Lenain *et al.*, 1984).

Androstenedione is a ZF cell product and is not produced directly by ZR cells. ZF cells do not produce DHEA because their high 3β-HSD activity does not permit the production of any Δ^5 steroids as endproducts (Figure 3). All endproduct ZF steroids are produced via the Δ^4 pathway. CYP17 protein expressed in heterologous cell systems such as COS cells has a much lower level of lyase activity on Δ^4 substrates than on Δ^5 substrates (Zuber *et al.*, 1988; Swart *et al.*, 1989). Consequently, most 17α-hydroxyprogesterone in the ZF cell is converted to deoxycortisol and cortisol, and only a small amount is converted to androstenedione. DHEA does not serve as a source of androstenedione within the adrenal gland. DHEA from the reticularis would have to move back to the fasciculata to be acted on by 3β-HSD, but the direction of blood flow opposes this possibility.

The absence of a change in the adrenal production of androstenedione makes it unlikely that adrenarche involves changes in the $C_{17,20}$-lyase activity of CYP17 (see Figure 3), as has been suggested on the basis of the observed modulation of lyase activity by phos-phorylation of CYP17 (Zhang *et al.*, 1995). Observations that have been used to deduce a change in adrenal lyase based on circulating levels of androstenedione,

DHEA, and other steroids (e.g. Kelnar and Brook, 1983) are misleading because these steroids are the products of different cell types. Changes in the ratios of these compounds may result from changes in the numbers of one cell type versus another, rather than tissue-wide changes in enzymatic activity. The implications of this analysis for clinical and basic science studies are, first, that questions regarding DHEA(S) biosynthesis need to be addressed in terms of adrenal biology, i.e., the birth, life, and death of ZR cells; and, second, that the major focus for the biochemical basis of DHEA(S) biosynthesis should be on the type II 3β-HSD gene and the presently unknown factors that suppress its expression in the ZR cell.

THE AGE-RELATED DECLINE IN DHEA BIOSYNTHESIS

The Loss of ZR Cells

The decline in plasma DHEAS from age 25 to age 70 (Figure 2) is the steepest continuous change in the endocrine system in humans over this age range (Orentreich *et al.*, 1984; Birkenhager-Gillesse *et al.*, 1994). The long-term aim of research on the age-related decline is to account for the steep decline in adrenal biosynthesis of DHEA(S), to clarify the effects of aging per se versus the effects of ill health, and to establish whether the age-related decline has functional consequences. It shows a high inter-individual variability perhaps caused by the heterogeneity of life histories in aging individuals (Vermeulen, 1980; Adams, 1985; Orentreich *et al.*, 1992). The decline in DHEAS contrasts with the maintenance of plasma cortisol levels in aging. The rate of production of cortisol declines slightly, but this in response to an age-related decrease in metabolic clearance (Blichert-Toft, 1978; Wolfsen, 1982). Plasma cortisol levels rise to the same extent in young and old subjects after administration of ACTH or CRH, whereas the increase in DHEAS is much less in old subjects (Vermeulen *et al.*, 1982; Liu *et al.*, 1990).

The simplest hypothesis is that the decrease of DHEA(S) biosynthesis in aging results from a decrease in the number of functional ZR cells. Thus, it is important to document the changes in the reticularis in aging and to understand the factors that maintain or delete ZR cells. Unfortunately it is not possible to correlate, within a single individual, the decline in DHEAS levels in aging with a decrease in ZR cells. In cross-sectional studies, there is a large, but variable, decline in DHEAS and a variable decrease in the width of the reticularis (Kreiner and Dhom, 1979; Parker *et al.*, 1997). The hypothesis that loss of DHEA(S) biosynthesis results from loss of ZR cells presumes that there is no feedback mechanism whereby levels of DHEAS regulate the width of the reticularis. Without a feedback mechanism, a loss of cells occurs without triggering a compensatory increase in the stimulus to DHEA(S) synthesis (for a more complete discussion see Hornsby, 1996).

Are ZR Cells Primed for Apoptosis?

An attractive hypothesis is that differences in gene expression between ZF cells and ZR cells cause ZR cells to be primed to undergo apoptosis and/or to be eliminated by the immune system. Cell loss may be the result of normal cell turnover or may occur during occasional periods of ischemia or other injury to the gland (see section below).

ZR cells but not ZF cells express MHC (major histocompatibility) class II antigens on their cell surface (Khoury *et al.*, 1987; Khoury and Berline, 1988). MHC class II molecules are expressed by a wide variety of normal tissues in vivo, as well as in abnormal conditions such as autoimmune diseases and tumors (Guardiola and Maffei, 1993). Cells expressing class II genes do not generally act as antigen-presenting cells because they lack the cofactors for antigen presentation (Guardiola and Maffei, 1993). It has been suggested that class II antigen expression on ZR cells results from effects of cytokines released by macrophages within the adrenal gland (Ehrhart-Bornstein *et al.*, 1996). However, class II expression persists in ZR cells in culture, even though they are mixed with ZF cells which do not become class II-positive (Khoury *et al.*, 1987). Thus it is likely that class II expression is a part of the differentiated phenotype of the ZR cell. Fas protein (CD95) is also expressed at a greater level on ZR cells (Wolkersdorfer *et al.*, 1996a; Wolkersdorfer *et al.*, 1996b). Cells expressing Fas protein may be killed by Fas ligand or by anti-Fas antibodies (Nagata and Golstein, 1995).

Intriguingly, ovarian corpus luteum cells also express MHC class II and Fas (Khoury and Marshall, 1990; Benyo and Pate, 1992; Quirk *et al.*, 1995). Like ZR cells, luteal cells differentiate postnatally, and are therefore "new" to the immune system. The corpus luteum is destined to be eliminated in the normal process of luteolysis (Pate, 1994). Perhaps ZR cells are similarly marked for deletion and/or intrinsic apoptosis. This hypothesis is in accord with the concept that the function of ZR cells is in the period of adrenarche to young adulthood, and perhaps the cells are no longer needed beyond this phase of the human life span. Thus, the decline in DHEA(S) production in aging may be a consequence of the death of ZR cells combined with the lack of a mechanism for their replacement, consistent with the idea that the reticularis is a "temporary" tissue.

Apoptosis, DNA Damage, p53 and p21 in the Adrenal Cortex in Vitro and in Vivo

In addition to normal cell turnover, periodic injury of the adrenal gland may, over a life time, cumulatively result in the loss and non-replacement of ZR cells. In humans and experimental animals, the adrenal cortex is susceptible to damage under a variety of pathophysiological conditions, including ischemia and sepsis (Bohm, 1982; Siegel *et al.*, 1994; Painter, 1994; Rao, 1995). The ultimate result of damage to the adrenal cortex resulting from various initiating events in human patients is massive hemorrhage of the adrenal glands leading to adrenal insufficiency and possibly death. Lower levels of damage, presumably involving similar pathogenetic mechanisms, result in derangement of structure and function of the adrenal cortex, associated with declines in plasma DHEAS (Kuhajda and Hutchins, 1979; Semple *et al.*, 1987; Lindh *et al.*, 1992), which may result from damage to the reticularis.

In this laboratory we studied the susceptibility of adrenocortical cells in vivo to DNA damage and apoptosis under varying conditions (Didenko *et al.*, 1996; Didenko *et al.*, 1999). In adrenocortical cells in glands from some organ donors, evidence of DNA damage was accompanied by immunoreactive p53 and $p21^{WAF1/CIP1/SDI1}$ (Didenko *et al.*, 1996). Adrenal gland injury was associated with events during trauma suffered by the patients before organs were removed for transplantation. DNA damage, present as single-strand breaks, caused activation of p53, which transcriptionally induced p21 (El-Deiry *et al.*, 1993). This process was accompanied by some apoptosis, presumably also mediated

by p53 (Canman *et al.*, 1994). Expression of p21 was noted in some apoptotic cells but does not appear to cause apoptosis (Didenko *et al.*, 1996).

We hypothesized that damage to the adrenal glands in human trauma victims would involve periods of low perfusion alternating with periods of higher blood flow, as observed in clinical practice (Siegel *et al.*, 1994; Painter, 1994; Rao, 1995). We observed that DNA damage, p53 and p21 could be induced in the rat adrenal gland by ischemia/reperfusion and by sepsis (Didenko *et al.*, 1996; Didenko *et al.*, 1999). A likely common element in injury of the adrenal gland is damage to the microvasculature. The function of capillary endothelial cells is altered by cytokines that circulate at high levels in shock and sepsis. The endothelium becomes leaky; extravasation of red blood cells and leukocytes is observed (Hinshaw, 1996). Although we have no direct evidence for this mechanism, it would account for DNA damage by exposure of the cells to oxygen-radical generating systems, such as granulocytes or transition metal/protein complexes (Griffin, 1981; Baehner *et al.*, 1982; Halliwell and Gutteridge, 1986). Oxygen radicals and their products cause extensive strand breaks in target cell DNA (Imlay and Linn, 1988).

In our studies, the extent of apoptosis in adrenal glands from organ donors was not sufficient to determine if it was more frequent in the reticularis than in other zones. However, if the hypothesis is correct that the reticularis selectively lacks a feedback mechanism for increasing its width when cells are lost, cell death anywhere in the cortex may result in a decreased width of the reticularis by shifting of the boundaries between the zones and efficient feedback restoration of the fasciculata (Hornsby, 1996).

CONCLUSIONS

The biosynthesis of DHEA(S) presents a major challenge for the biologist concerned with the human endocrine system, because the cell type responsible, the zona reticularis cell, is confined to humans and closely related primates. The appearance of DHEAS in plasma in late childhood and its disappearance with age presents a puzzle that lacks satisfactory biological explanations, although the concept that the age-related decline results from death and non-replacement of the cells of the zona reticularis forms a working hypothesis on which to base future studies.

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MODIFICATION OF MATERIALS FORMED FROM POLY(L-LACTIC ACID) TO ENABLE COVALENT BINDING OF BIOPOLYMERS: APPLICATION TO HIGH-DENSITY THREE-DIMENSIONAL CELL CULTURE IN FOAMS WITH ATTACHED COLLAGEN

JI ZHENG, S. ROBERT NORTHRUP, AND PETER J. HORNSBY[1]

Huffington Center on Aging and Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

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SUMMARY

We describe a method for increasing the hydrophilicity of materials formed from biodegradable polymers and introducing chemical functional groups on their surfaces. Poly(L-lactic acid) was blended with poly(ε-CBZ-L-lysine) at an 80:20 ratio. Films of the mixture were prepared and foams were made by solvent casting and salt leaching. Amino groups on the surface of the polymer mixture were deprotected by acid hydrolysis. As an example of the applicability of the technique for attachment of biomolecules, we covalently linked collagen to the deprotected amino groups, creating a surface capable of high density growth of a differentiated cell type (bovine adrenocortical cells). The method should be generally useful for surface modification of biodegradable polymer materials used in tissue engineering.

Key words: biodegradable polymers; tissue engineering; adrenocortical cells; poly-L-lysine.

INTRODUCTION

An essential component of tissue engineering is the construction of biocompatible, degradable scaffolds, which can provide temporary support of cells during vascularization and the formation of a functional tissue structure. Scaffolds can be formed from synthetic polymers that can be produced in various physical forms and their properties can be tightly controlled. However, their surfaces usually do not provide for efficient cell attachment and growth. Desirable features of polymers used to form scaffolds would be the provision on their surfaces of biosignaling molecules, such as cell adhesion proteins, growth factors, or cytokines that may be critical for proper cellular function (9,10,24).

Poly(α-hydroxy ester) polymers such as poly(L-lactic acid) (PLLA) are biocompatible, degrading to products that can be eliminated by metabolism or renal excretion (6). PLLA foams and woven fibers have been used in cell transplantation experiments as scaffolds for guided tissue regeneration (4,7,20). However, these polymers are highly crystalline and hydrophobic, and it is desirable to impart hydrophilicity and the ability for chemical modification to these polymers by introducing functional groups. Several new biodegradable copolymers with molecules providing functional groups have been described (1,5,13,21). Here we describe a general method for increasing the hydrophilicity and introducing chemical functional groups in materials formed from biodegradable polymers. We demonstrate the applicability of the technique for the attachment of biomolecules by using collagen, thereby creating a surface capable of high density growth of a differentiated cell type.

[1]To whom correspondence should be addressed at Huffington Center on Aging, Baylor College of Medicine, 1 Baylor Plaza M320, Houston, Texas 77030.

MATERIALS AND METHODS

Preparation of polymer materials. Films were prepared by evaporation of polymer solutions, and porous foams were prepared by solvent casting and salt leaching (22). 240 mg poly(L-lactic acid) (PLLA, mol. wt. 100 000, Polysciences, Warrington, PA) and 60 mg poly(ε-CBZ-L-lysine) (PCBZL, mol. wt. 260 000, Sigma Chemical Co., St. Louis, MO) were dissolved in 10 ml chloroform. For preparation of polymer films, 300 μl of the polymer solution was placed on the surface of a 2-cm² circular glass coverslip. The solvent was allowed to evaporate for 24 h at room temperature and then the film was dried under a vacuum at room temperature for 3 h. The film (~100 μm thick) was removed from the coverslip by immersion in water. The film circle was trimmed to fit the bottom of a well (1.76 cm²) of a 24-well culture plate. For the preparation of polymer foams, 2 ml polymer solution was placed in a 5-cm glass petri dish. The solvent was allowed to evaporate from the covered petri dish for 24 h at room temperature. Residual solvent was removed by vacuum drying for 3 h. The dried PLLA/PCBZL mixed polymer films (~100 μm thick) were used as the base for production of porous foams (~250 μm thick) as follows. Two milliliters of polymer solution was added to the surface of the solid polymer. One gram of salt particles sieved to less than 150 μm through polypropylene mesh (Spectrum Laboratory Products, Los Angeles, CA) (22) was immediately added to the surface of the polymer solution and care was taken to ensure that the particles were evenly distributed. The solvent was allowed to evaporate from the covered petri dish over a 48-h period. Residual solvent was removed by vacuum drying for 3 h. Salt was leached from the foams by immersion in 250 ml water, with frequent changes, for 24 h. Films and foams were then subjected to the chemical modifications described in the next section or were used directly in cell culture experiments, as described below.

Surface deprotection of lysine ε-amino groups. PLLA/PCBZL mixed polymer films or foams were placed in 4.3 *M* hydrochloric acid in glacial acetic acid for 30 min at 37° C with shaking. The acid was neutralized with 0.1 *M* sodium carbonate overnight. The polymer was then washed with water until the pH reached 7.0, and was stored at 4° C, without drying.

The concentration of deprotected amino groups on the polymer surface was determined by reaction with fluorescamine (10 mg/ml) in chloroform. The fluorescence was measured with excitation less than 360 nm and emission greater than 418 nm (23).

Covalent binding of collagen. A solution of type I collagen was prepared by dissolving rat tail collagen fibers in 12 m*M* hydrochloric acid (18). After

a 48-h incubation of the fibers in HCl at 4° C, insoluble material was removed by centrifugation at 10 000 × g for 1 h. Surface-deprotected polymer materials were reacted with 5 mg water-soluble carbodiimide (1-ethyl-3-[3-bimethylaminopropyl]carbodiimide) per ml and 1 mg collagen per ml in 10 m*M* 2-(*N*-morpholino)ethane sulfate (MOPS), pH 4.5. The reaction was allowed to proceed for 48 h at 4° C. Each film or foam disk was washed five times with 5 ml 12 m*M* HCl, twice with 5 ml 20 m*M* sodium hydroxide, and then extensively with water (10 changes of 5 ml each).

To determine the quantity of collagen attached to the surface, collagen was fluorescently labeled by reaction of 0.5 mg collagen per ml with 1.5 mg fluorescein isothiocyanate (FITC) per ml at 4° C for 24 h. After reaction, the FITC-labeled collagen was separated from free FITC by gel filtration with cellulose beads (80–100 μm, exclusion range 10 000–1 000 000). Films were incubated in a collagen solution, as described above, containing 10% FITC-labeled collagen. We determined attached collagen by dissolving the film in chloroform and measuring fluorescence at greater than 550 nm with excitation less than 418 nm.

Water contact angle. Advancing and receding contact angles were measured with static drops of phosphate buffer (pH 7.0) in a Rame-Hart model 100 goniometer. Contacting aqueous solutions were applied (for advancing angles) and withdrawn (for receding angles) with a Matrix Technologies Electrapipette operated at the slowest speed (1 μl/s). With the pipette tip touching the drops, two measurements of the contact angle were performed on opposite edges of four drops for each polymer surface.

Cell culture. Bovine adrenocortical cells were cultured according to previously published procedures (8). Primary cultures were derived by enzymatic and mechanical dissociation of freshly obtained tissue (3-h incubation with 1 mg type I-A collagenase per ml and 0.1 mg DNase per ml). Cells were grown in Dulbecco's Eagle's Medium/Ham's F-12 1:1 with 10% fetal bovine serum, 10% horse serum, and 0.1 ng recombinant basic fibroblast growth factor (FGF) (8).

For assessment of cell attachment and growth, polymer films and foams of the sizes described above were sterilized by brief immersion in 70% ethanol, followed by rinsing in sterile water. For measurement of cell attachment, films were seeded with 2×10^5 bovine adrenocortical cells in 200 μl medium. For measurement of cell growth, films were seeded with 10^4 cells in 1 ml medium. Foams were seeded with 1.5×10^5 cells in 5 ml medium. For measurement of cell attachment, after 6 h polymer films were rinsed with phosphate-buffered saline (PBS), and the attached cells were fixed in methanol (19). For cell counting, the films with fixed cells were incubated for 5 min in 10 μg of the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) per ml. Attached cells were counted by fluorescence microscopy. Ten randomly selected microscope fields (each 0.88 mm^2) were counted. For cell growth experiments, polymer films and foams with attached adrenocortical cells were incubated in the medium described above at 37° C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 (8) for 3 or 4 d. For measurement of cell growth, films were fixed and stained for counting of cells by fluorescence microscopy as described above.

Additionally, fragments of polymer foams were prepared for scanning electron microscopy (SEM). Samples were fixed in 3% glutaraldehyde followed by 1% osmium tetroxide, and dehydrated in graded acetone. The samples were subjected to critical-point drying, coated with platinum and lead, and observed by SEM (Jeol JEM-100c)

Sources of chemicals. MOPS, FITC, fluorescamine, cellulose beads, water-soluble carbodiimide and DAPI were from Sigma Chemical Co., St. Louis, MO. Cell culture components were from Life Technologies, Gaithersburg, MD. All other chemicals and materials used were from Fisher Scientific Co., Pittsburgh, PA.

RESULTS

Preparation of polymer blends with surface amino groups for attachment of biopolymers. To provide a surface suitable for covalent attachment of biopolymers, we mixed PLLA with poly(CBZ- L-lysine) (PCBZL) in an 80:20 ratio. CBZ groups are used during peptide synthesis to prevent the participation of side chain amino groups in -CONH- bond formation (12). The CBZ-protected form of polylysine has a hydrophobicity appropriate for blending with PLLA in organic solvents. Following solvent removal, amino groups of lysine residues exposed on the surface of the polymer were deprotected by acid hydrolysis (2). The greatest increase in amino groups was produced when deprotection was performed with 4.3 *M* HCl in glacial acetic acid (Fig. 1). PLLA/PCBZL mixtures deprotected in this way were used in subsequent experiments.

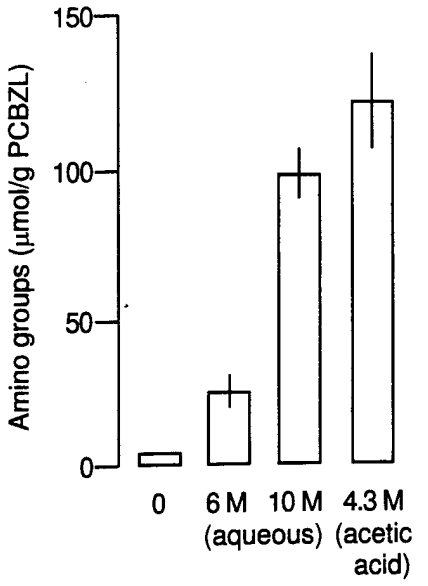

FIG. 1. Increase of amino groups on the surface of films formed from an 80:20 PLLA/PCBZL mixture after deprotection with acid. Averages and standard deviations are given; n = 3.

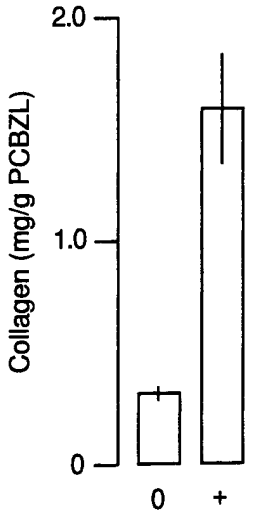

FIG. 2. Binding of collagen (1 mg/ml) to deprotected PLLA/PCBZL by adsorption and covalent attachment (± carbodiimide). Averages and standard deviations are given; n = 4.

Collagen is an example of a biomolecule that is predicted to improve the properties of synthetic polymers with respect to cell attachment and growth. We tested the attachment of collagen to the surface of deprotected PLLA/PCBZL. A small amount of collagen was bound by noncovalent adsorption, but ~fourfold more could be covalently attached by cross-linking with carbodiimide (Fig. 2).

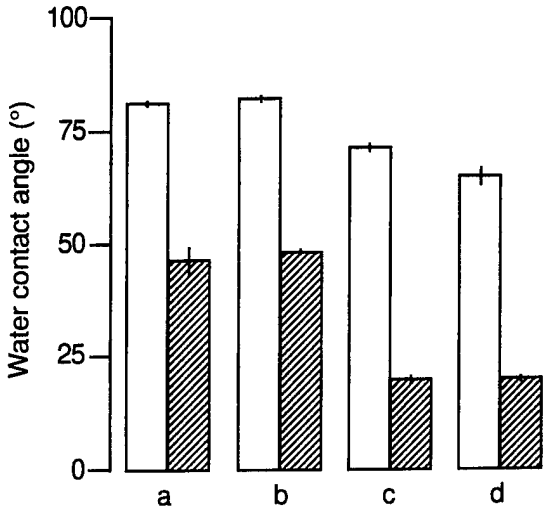

FIG. 3. Advancing (*open columns*) and receding (*hatched columns*) contact angles of films formed from different polymers. (*a*) Unmodified PLLA; (*b*) PLLA/PCBZL mixture; (*c*) deprotected PLLA/PCBZL; and (*d*) deprotected PLLA/PCBZL with covalently attached collagen. Averages and standard deviations are given for four measurements for each.

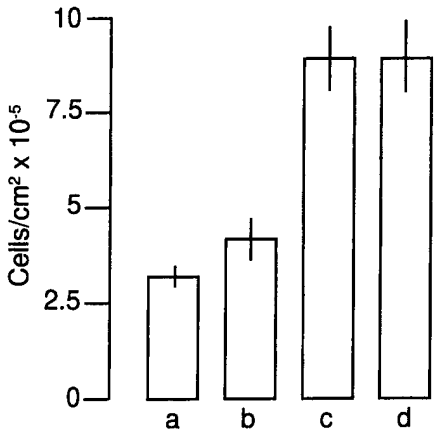

FIG. 4. Attachment of primary bovine adrenocortical cells to polymer films. Cells were plated on the surface of films in culture medium and were allowed to attach for 6 h. Attached cells were counted with fluorescence microscopy. (*a*) Unmodified PLLA; (*b*) PLLA/PCBZL mixture; (*c*) deprotected PLLA/PCBZL; and (*d*) deprotected PLLA/PCBZL with covalently attached collagen (n = 6 for each).

The properties of the surfaces of films formed from the modified polymers were tested by measurement of the water contact angle. The lowered water contact angle of films formed with deprotected PLLA/PCBZL and of films with covalently attached collagen demonstrates the increased hydrophilicity of these modified polymer surfaces (Fig. 3).

To assess the surfaces of materials formed from these polymers as substrata for cell growth, we tested the ability of films to support the attachment and growth of a differentiated cell type, primary bovine adrenocortical cells. A greater percentage of cells were able to attach to deprotected PLLA/PCBZL and to PLLA/PCBZL with bound collagen than to PLLA or PLLA/PCBZL (Fig. 4).

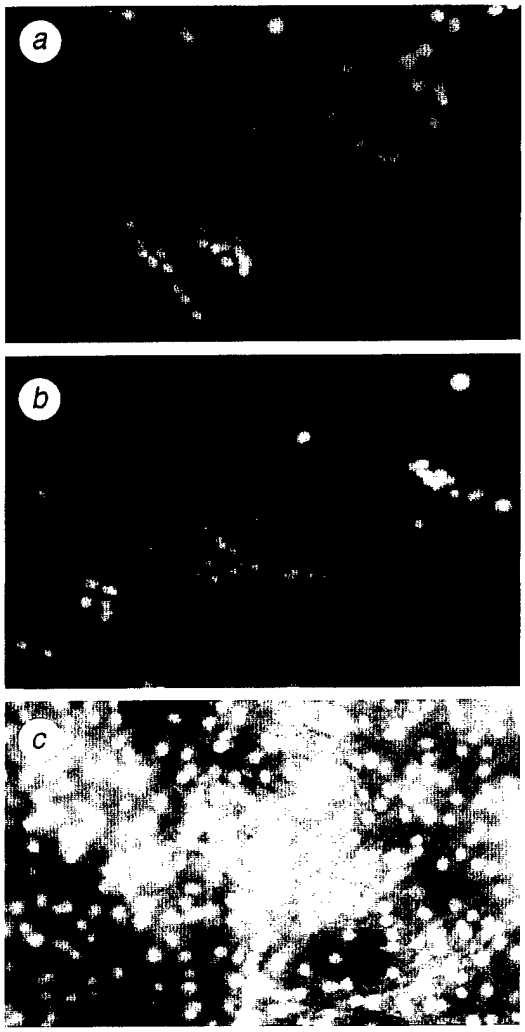

FIG. 5. Cell growth on polymer films. Primary bovine adrenocortical cells were plated on the surface of films and were allowed to grow for 3 d. Cells were then fixed, stained by the DNA-binding dye DAPI, and photographed by fluorescence microscopy. (*a*) Cells plated on unmodified PLLA; (*b*) cells plated on PLLA/PCBZL mixed polymers; and (*c*) cells plated on deprotected PLLA/PCBZL with covalently attached collagen.

Following attachment, cells grew more rapidly on the modified polymer films. The deprotected polymer, especially with covalently attached collagen, was able to support a much higher cell density than untreated PLLA/PCBZL or unmodified PLLA. Fig. 5 shows the microscopic appearance of cells growing on the polymer films and Fig. 6 shows the cell density after 4 d of growth on the films.

Properties of mixed polymer foams. PLLA/PCBZL porous foams were prepared by solvent casting and salt leaching (22). As assessed qualitatively by SEM, pores were of relatively uniform morphology and evenly distributed (Fig. 7). By SEM most pores appeared to be interconnected. No changes in the surface structure visible by SEM were produced by deprotection hydrolysis or by covalent attachment of collagen.

To provide another measure of the hydrophilicity of the polymer surfaces, in addition to water contact angle, foams were immersed in water and weighed at intervals to assess the water intrusion volume

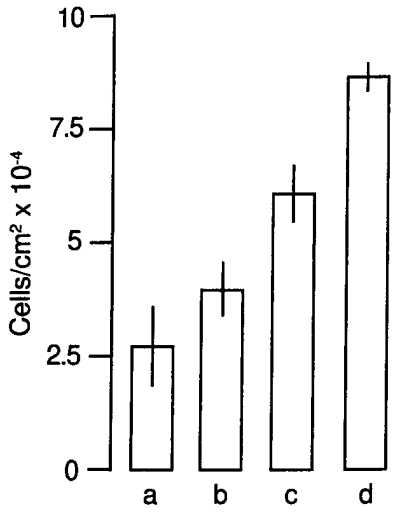

FIG. 6. Cell density after growth on polymer films. Primary bovine adrenocortical cells were plated on the surface of films and were allowed to grow for 4 d. We assessed cell density by counting cells under fluorescence microscopy after fixation and staining with DAPI. (*a*) Unmodified PLLA; (*b*) PLLA/PCBZL mixture; (*c*) deprotected PLLA/PCBZL; and (*d*) deprotected PLLA/PCBZL with covalently attached collagen (n = 6 for each).

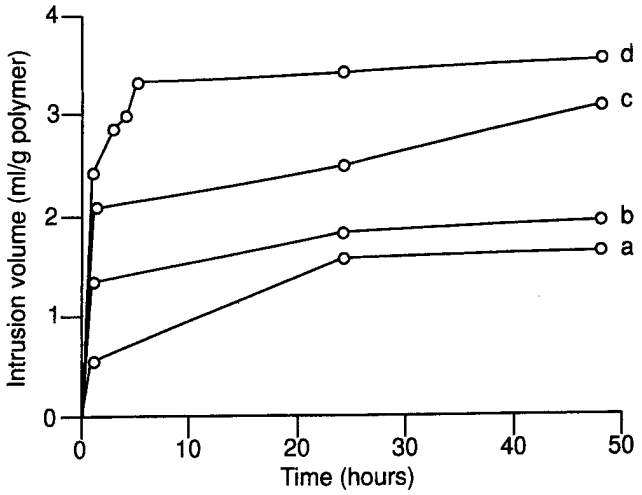

FIG. 8. Water intrusion volume of different polymer foams. Disks of 5-cm diameter of the four foams were weighed when dry and then weighed at the indicated intervals after immersion in water, removal, and careful blotting of excess water from the surface of the disks. The increase in weight gives the water intrusion volume. (*a*) PLLA; (*b*) PLLA/PCBZL mixture; (*c*) deprotected PLLA/PCBZL; and (*d*) deprotected PLLA/PCBZL with covalently attached collagen.

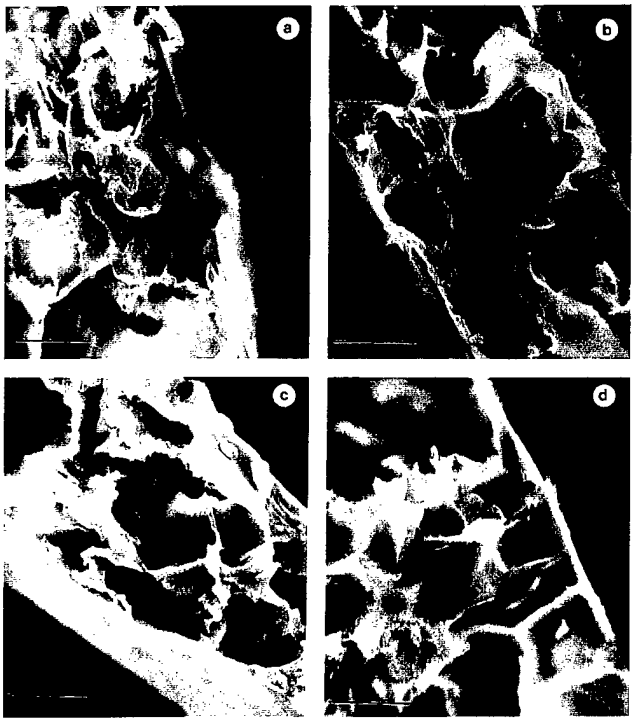

FIG. 7. SEM images of porous polymer foams. (*a*) PLLA; (*b*) PLLA/PCBZL mixture; (*c*) deprotected PLLA/PCBZL; and (*d*) deprotected PLLA/PCBZL with covalently attached collagen. *Bar* = 100 μm.

(17) (Fig. 8). Modified PLLA/PCBZL and modified PLLA/PCBZL with attached collagen showed enhanced hydrophilicity in comparison with unmodified PLLA/PCBZL or PLLA.

Growth of cells in mixed polymer foams. To assess the suitability of polymer foams with modified surfaces for the growth of a differentiated cell type, foams were seeded with bovine adrenocortical cells and were allowed to grow for 3–5 d. Visualization of cell growth by DAPI fluorescence and by SEM (Figs. 9 and 10) showed good cell attachment and growth on foams formed from deprotected PLLA/PCBZL and deprotected PLLA/PCBZL with collagen. On the latter two polymers, cells were spread out and formed a monolayer, and could also be observed to have penetrated deep into the foam (Fig. 9 *c* and *d*).

DISCUSSION

Deprotection of the protected form of poly(L-lysine), PCBZL, generates amines that provide an improved surface for cell growth and which can be used for derivatization of the surface of materials, such as PLLA, into which the polymer has been mixed. It was previously shown that attachment of polylysine, fibronectin, and insulin on surface-hydrolyzed poly(methylmethacrylate) greatly enhanced cell adhesion (25). Enhancement of cell attachment and growth by amino groups results from an increase of electrostatic interaction of the cell surface with the polymer combined with the effect of increased adsorption of proteins from the growth medium (11,14,15,16,25). We used collagen as an example of a biopolymer that can be attached to deprotected PCBZL. Collagen improves cell attachment, growth, and function of polymers used in tissue engineering (3) and it supported excellent attachment and growth of the differentiated cells used in these experiments.

PLLA/PCBZL mixtures used in these experiments were found to be suitable for the production of porous foams, which had increased

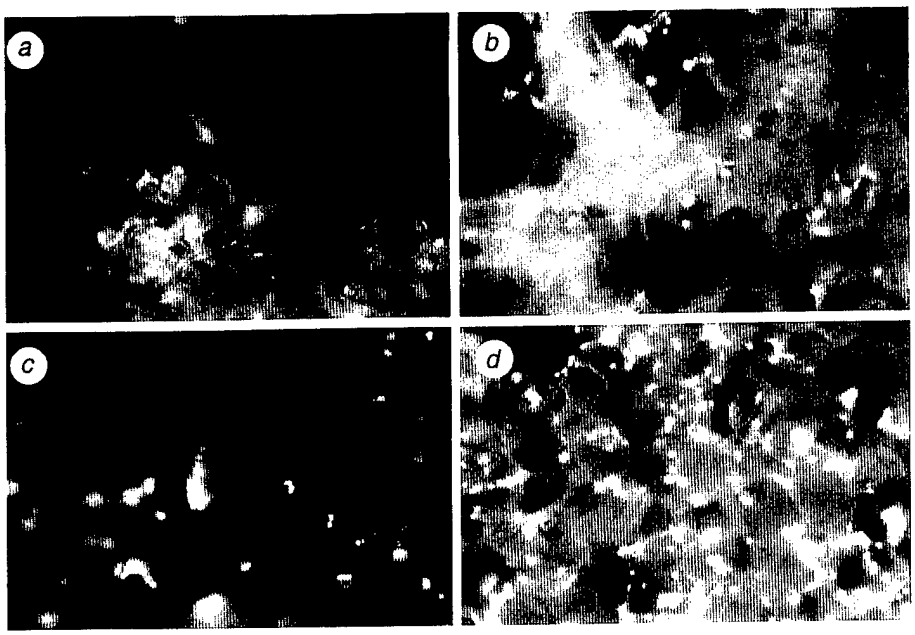

FIG. 9. Cell growth in porous polymer foams. Primary bovine adrenocortical cells were seeded into the foams and were allowed to grow for 5 d. Cell density was assessed by fluorescence microscopy with DAPI. (*a*) Unmodified PLLA foam; (*b*) PLLA/PCBZL mixed polymer foam; (*c*) deprotected PLLA/PCBZL foam; and (*d*) deprotected PLLA/PCBZL foam with covalently attached collagen.

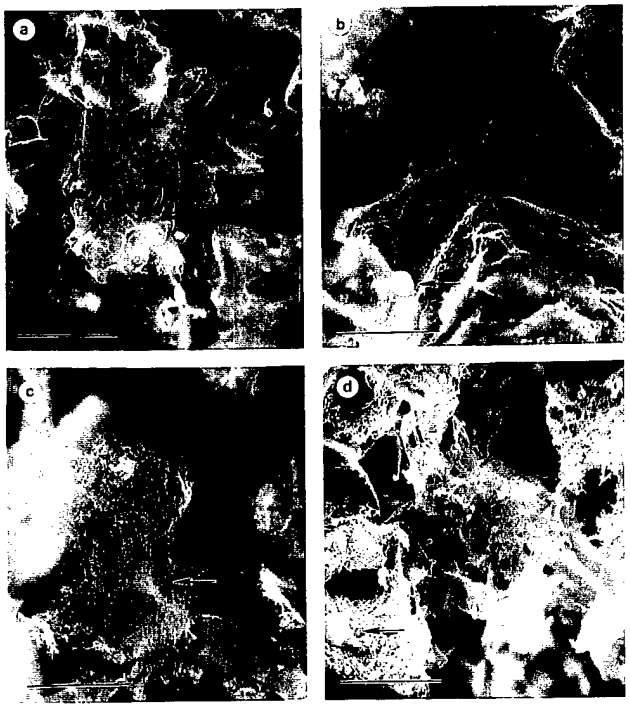

FIG. 10. SEM images of cells attached to porous polymer foams. Primary bovine adrenocortical cells were seeded into the foams and were allowed to grow for 3 d. (*a*) Unmodified PLLA foam; (*b*) PLLA/PCBZL mixed polymer foam; (*c*) deprotected PLLA/PCBZL foam; and (*d*) deprotected PLLA/PCBZL foam with covalently attached collagen. *Arrows* indicate cells. *Bar* = 50 μm.

hydrophilicity but were structurally similar to foams formed from unmodified PLLA. Foams have a greatly increased available surface area for cell attachment and growth, and support high-density growth of primary cells.

The development of functionalized polymer materials blended with PLLA should offer new opportunities for tissue engineering by providing for the attachment of a wide variety of biosignaling molecules.

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Transplantation of primary bovine adrenocortical cells into *scid* mice

Michael Thomas, Peter J. Hornsby *

Huffington Center on Aging and Department of Cell Biology, Baylor College of Medicine, 1 Baylor Plaza, M320 Houston, TX 77030, USA

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Abstract

Bovine adrenocortical cells were transplanted into *scid* mice, using a small cylinder inserted beneath the kidney capsule. The tissue formed from primary bovine adrenocortical cells replaced the essential functions of the animals' own adrenal glands, which were removed during the cell transplantation procedure. Most adrenalectomized animals bearing transplanted cells survived indefinitely, whereas adrenalectomized control animals died following surgery. Formation of well-vascularized tissue at the site of transplantation was associated with stable levels of cortisol in the blood, replacing the mouse glucocorticoid (corticosterone). Ultrastructurally, the cultured cells before transplantation had characteristics of rapidly growing cells, but tissue formed in vivo showed features associated with active steroidogenesis. We investigated two potentially critical aspects of the procedure: the provision of support for angiogenesis in the transplant by the inclusion of FGF-secreting 3T3 cells with the adrenocortical cells; and the administration of synthetic steroids as a temporary replacement for steroids lost by adrenalectomy. We found that FGF was required for the rapid formation of well-vascularized tissue, whereas steroid administration avoided some early mortality but was not absolutely required. In contrast to transplants formed from clonal cells, which did not usually secrete aldosterone, transplants formed from primary bovine adrenocortical cells, even though derived from the zona fasciculata, secreted aldosterone as well as cortisol.

Keywords: Transplantation; Bovine adrenocortical cells; *Scid* mice; Fibroblast growth factor; Adrenocortical steroidogenesis; Adrenal zonation

1. Introduction

Cell transplantation is a powerful new technique for addressing a variety of questions in cell and molecular biology of endocrine systems (Ricordi and Starzl, 1991; Langer and Vacanti, 1993; Vogt et al., 1994; McKay, 1997; Rando and Blau, 1997; Gage, 1998). In a previous study, we transplanted clonal bovine adrenocortical cells into *scid* mice, using a small cylinder inserted beneath the kidney capsule (Thomas et al., 1997). The tissue formed from clonal bovine adrenocortical cells was functional, supported the life and health of the adrenalectomized animals, and resembled normal adrenocortical tissue histologically and ultrastructurally. Most of the experiments used a single clone of bovine adrenocortical cells, but we also showed that five of 20 freshly isolated bovine adrenocortical cell clones could form vascularized functional tissue after transplantation. These experiments demonstrated for the first time that an endocrine tissue, replacing the host animal's organ, can be derived from a single normal somatic cell.

In the present experiments, we expanded the scope of our studies of the transplantation of adrenocortical cells by investigating two potentially critical aspects of the procedure. First, we studied the role of fibroblast growth factor (FGF) supplied to the transplant by the inclusion of FGF-secreting 3T3 cells with the adrenocortical cells. Additionally, we investigated the role of synthetic steroids administered to the animals over the first week following transplantation as a temporary replacement for the steroids lost due to the adrenalectomy.

* Corresponding author. Tel: +1-713-7987334; fax: +1-713-7986688.

E-mail address: phornsby@bcm.tmc.edu (P.J. Hornsby)

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Table 1
Groups of animals with transplanted bovine adrenocortical cells[a]

A	7 days steroids	4×10^5 mitomycin C-treated FGF-secreting 3T3 cells	$n = 9$
B	No steroids	4×10^5 mitomycin C-treated FGF-secreting 3T3 cells	$n = 15$
C	3% sodium chloride	4×10^5 mitomycin C-treated FGF-secreting 3T3 cells	$n = 4$
D	7 days steroids	No 3T3 cells	$n = 5$
E	No steroids	No 3T3 cells	$n = 11$
F	7 days steroids	4×10^5 irradiated FGF-secreting 3T3 cells	$n = 10$

[a] Groups of animals receiving bovine adrenocortical cells in this study are listed. All animals received 2×10^6 primary bovine adrenocortical cells. Where indicated, dexamethasone and fludrocortisone were given for 7 days as described in Section 2, or a solution of sodium chloride was made available in a separate water bottle. The number of cotransplanted FGF-secreting 3T3 cells, treated as described, is indicated. The number of animals in each group is shown.

2. Methods

2.1. Growth of bovine adrenocortical cells in culture

Bovine adrenocortical cells were derived by enzymatic and mechanical dispersion from the adrenal cortex of 2-year-old steers, as previously described (Hornsby and McAllister, 1991; Hornsby, 1994). Primary cell suspensions were stored frozen in liquid nitrogen. Frozen cells were thawed and replated in Dulbecco's Eagle's Medium/Ham's F-12 1:1 with 10% fetal bovine serum, 10% horse serum and 0.1 ng/ml recombinant FGF-2 (Mallinckrodt, St. Louis, MO) (Hornsby and McAllister, 1991; Hornsby, 1994). Cells were grown in culture for 7 days before transplantation.

2.2. FGF-transfected 3T3 cells

A line of 3T3 cells stably expressing FGF-1 fused in frame with a signal peptide from hst/KS3, yielding a highly angiogenic secreted product (Forough et al., 1993), was generously supplied by T. Maciag. 3T3 cells were grown under the same conditions as bovine adrenocortical cells. To render the cells incapable of further division after transplantation, they were treated with mitomycin C (Thomas et al., 1997) or were lethally irradiated. Cells were incubated at ~20% confluence for 24 h with 2 μg/ml mitomycin C (Sigma, St. Louis, MO) or were pelleted and exposed to 60 Gray radiation from a ^{137}Cs source (Gammacell 1000 model C, AECL Industrial, Kanaka, Ont.).

2.3. Transplantation of cells beneath the kidney capsule of scid mice

ICR *scid* mice originally purchased from Taconic (Germantown, NY) were maintained in an animal barrier facility as a breeding colony. Animals (both males and females) at an age greater than 6 weeks (~25 g body weight) were used in these experiments. Procedures were approved by the institutional animal care committee and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Under tribromoethanol anesthesia, mice were adrenalectomized and received transplanted adrenocortical cells in a single procedure. A longitudinal incision was made with fine scissors in the dorsal skin. A 1.5-cm incision in the lateral body wall was made to open the retroperitoneal space. Using two small forceps, the lumbar muscles were separated from the fat tissue above the adrenal gland. The forceps were used to isolate the adrenal gland and the pedicle was clamped with a fine hemostat. The pedicle was ligated with 6-0 surgical thread and severed. The integrity of the gland and thus the completeness of adrenalectomy was assessed under the dissecting microscope. Adrenalectomy was performed on both sides.

In order to confine the transplanted adrenocortical cells within a defined space so that the growth, vascularization and function of the cells could be readily studied, we used a small polycarbonate cylinder to create a virtual space beneath the capsule into which the cells could be introduced (Thomas et al., 1997). A

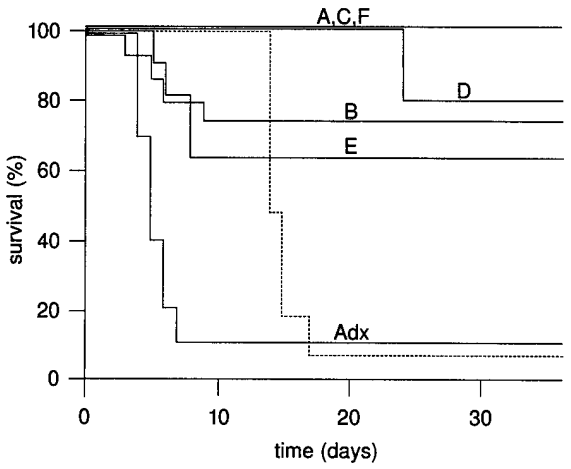

Fig. 1. Survival of mice after adrenalectomy and transplantation of primary bovine adrenocortical cells. The letters indicate the groups of animals listed in Table 1. Numbers of animals in each group are listed in the table. Adx = survival of adrenalectomized animals; the dashed line shows the previously determined (Thomas et al., 1997) survival of adrenalectomized animals without transplanted cells that received synthetic steroids for the first 7 days.

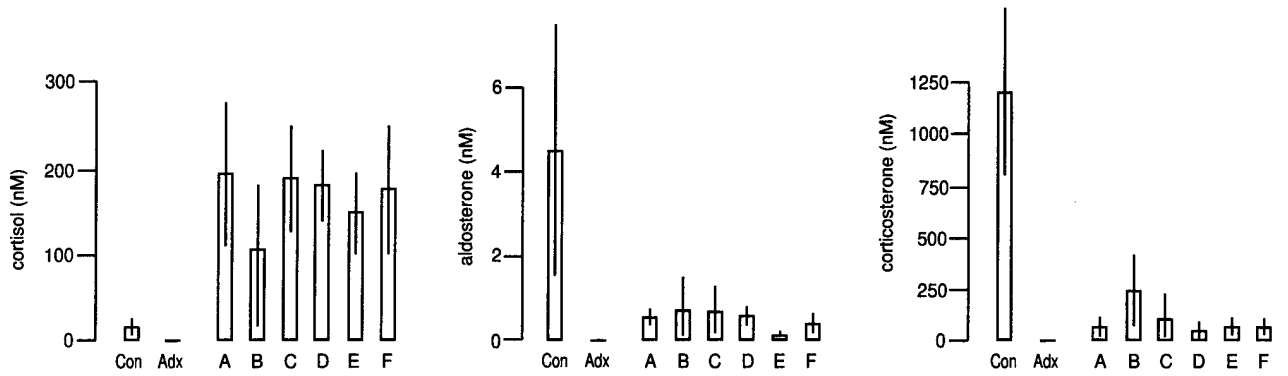

Fig. 2. Levels of plasma steroids in mice with transplanted adrenocortical cells, at the time of sacrifice at 36 days. The groups of animals are listed in Table 1; Con = nonadrenalectomized animals without transplants; Adx = adrenalectomized animals without transplants, 3 days following adrenalectomy. Average values ± SD.

1-mm length of polycarbonate tube (3-mm internal diameter) was surface-polished by brief exposure to dichloromethane vapor. Cylinders were sterilized in 70% ethanol prior to use.

Using the incision in the body wall prepared for adrenalectomy, the left kidney was exteriorized and a small transverse incision was made through the capsule on the ventral surface of the kidney near the inferior pole. Using one point of a fine forceps, a pocket was created under the capsule. A polycarbonate cylinder was pushed partially into the pocket under the capsule, filled with culture medium, and then introduced fully into the pocket so that the capsule on the top and the kidney parenchyma on the bottom formed a sealed space. Adrenocortical cells were introduced into this space as follows. Cells were released from the culture dish by digestion with bacterial protease (Hornsby and McAllister, 1991; Hornsby, 1994). Using 2×10^6 adrenocortical cells, either without 3T3 cells or mixed with 4×10^5 mitomycin C-treated or irradiated 3T3 cells were injected in each animal. The cell suspension was pelleted and kept on ice. For injection, the pellet

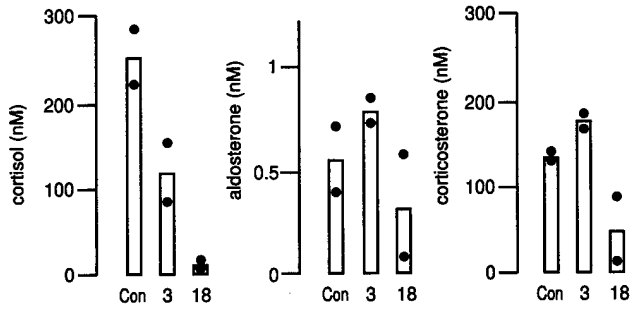

Fig. 3. Changes in plasma steroid levels after administration of an inhibitor of 17α-hydroxylase, 22-amino-23,24-bis-norchol-5-en-3β-ol (22-ABC). Mice bearing transplanted bovine adrenocortical cells received either 3 μg/g or 18 μg/g 22-ABC in three divided doses, injected subcutaneously, at 1-h intervals. Blood samples were then taken after one additional hour.

was resuspended in medium, the total vol. of the suspension being just greater than the vol. of the pelletted cells. The cells were introduced into the subcapsular cylinder by a transrenal injection using a 50 μl Hamilton syringe with a blunt 22 gauge needle. Following transplantation of the cells, the kidney was returned to the retroperitoneal space and bathed in ~3 ml PBS. The body wall was closed with 6-0 nylon sutures and the skin was closed with surgical staples. Animals were maintained at 35°C ambient temperature until recovery from the anesthetic.

Post-operative care for the animals consisted of the administration of synthetic steroids (for most animals; see Table 1) and the administration of analgesics and antibiotics in the drinking water. For 7 days after surgery, animals receiving hormonal support were injected subcutaneously once daily with 1 μg/g body weight fludrocortisone acetate and dexamethasone phosphate (Luo et al., 1994). Animals were given access to drinking water containing 1 mg/ml acetaminophen, 0.1 mg/ml codeine, 1 mg/ml tetracycline, 1 mg/ml sulfamethoxazole, and 0.1 mg/ml trimethoprim. The pH of the drinking water (6.05) was adjusted with potassium hydroxide so that, for most animals, additional sodium, beyond that already supplied in the diet, was not given via the drinking water. However, one group of animals was given access to sodium-containing drinking water instead of synthetic steroid support (see Table 1). These animals were supplied with two water bottles, one containing the antibiotic/analgesic mixture described above and one containing 3% sodium chloride in water. Mice voluntarily consumed ~2 ml of this solution per day.

Blood samples for assessment of circulating adrenal steroid levels were not routinely taken during the first 7 days because preliminary tests showed that adrenal steroidogenesis was suppressed by administration of steroids. One week after cessation of the steroid administration, and at approx. weekly intervals thereafter, tail

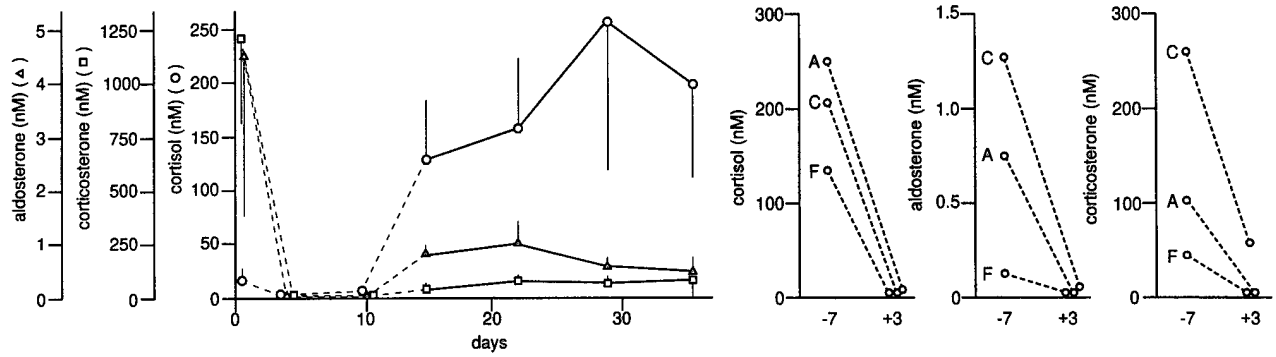

Fig. 4. Left, time course of changes in plasma cortisol and corticosterone in animals with transplanted bovine adrenocortical cells (group A of Table 1). The abscissa indicates the time in days since cell transplantation. Average values ± SD; (○) cortisol; (Δ) aldosterone; (□) corticosterone. Right, changes in plasma steroids after removal of the kidney with transplanted cells. Three animals were treated similarly to those in groups A, C, and F (Table 1) but were subjected to nephrectomy at day 36 and were killed after a further 3 days. The plasma steroid levels are shown 7 days before nephrectomy (−7) and at the time of sacrifice 3 days after nephrectomy (+3).

blood samples were taken 15 min after the injection of ACTH (Sigma, 0.01 units per g body weight). In most experiments animals were sacrificed 36 days after cell transplantation. Animals were injected with ACTH 15 min before sacrifice and cardiac blood samples were removed under anesthesia.

In some experiments, the kidney bearing the transplant tissue was removed in a second surgical procedure. The pedicle to the kidney was ligated with a 6-0 suture and the kidney was removed. The animals received the standard postoperative care, as described above, without steroid administration.

2.4. Histology and immunohistochemistry

The fixation, paraffin embedding and histological examination of tissue formed from transplanted cells was carried out using standard techniques. For visualization of nuclei by DNA-binding dye fluorescence, deparaffinized sections were incubated for 5 min at 4°C in 1 μg/ml 4′,6-diamidino-2-phenyl indole (DAPI). After washing, sections were observed and photographed by fluorescence microscopy.

Some tissue sections were stained for expression of the Ki-67 proliferation-associated antigen using monoclonal antibody MIB-1 (Immunotech, Westbrook, ME) and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA), as recommended by the manufacturers. This antibody does not recognize the mouse homologue of Ki-67 but recognizes the bovine protein with staining equivalent to that observed with human tissues (our unpublished observations). Sections were lightly counterstained with hematoxylin.

2.5. Electron microscopy

Tissue formed from transplanted cells, control adrenal glands, and cultured adrenocortical cells were fixed with 2.5% glutaraldehyde in PBS, post-fixed in osmium tetroxide, and embedded in Epon. Cultured cells were removed from the culture dish by scraping, after fixation but before embedding. Sections (100 nm thick) were stained with uranyl acetate and lead citrate and were examined and photographed by electron microscopy.

2.6. Radioimmunoassays

Radioimmunoassays were performed directly on plasma samples from animals with transplanted cells using the following kits. Cortisol: Corti-cote (ICN Pharmaceuticals, Orangeburg, NY). The manufacturer reports that this antibody has a cross-reaction of 1.2% with corticosterone. Corticosterone: ImmuChem double antibody kit (ICN). This antibody has a reported cross-reaction of 0.05% with cortisol. Aldosterone: Active Aldosterone kit (Diagnostic Systems Laboratories, Webster, TX). The manufacturer reports a cross-reaction of this antibody with corticosterone of 0.03% and no detectable cross-reaction with cortisol. We did further testing of possible cross-reactions of this antibody by adding steroids to samples of plasma from adrenalectomized animals without transplanted cells. The antibody showed $< 0.02\%$ cross-reaction with 18-hydroxycortisol and 0.5% cross-reaction with 18-oxo-cortisol (these steroids were a gift of S. Ulick).

3. Results

3.1. Survival of animals with transplanted cells and plasma steroid levels

Primary bovine adrenocortical cells were transplanted beneath the kidney capsule of *scid* mice, using a small polycarbonate cylinder to confine the newly

formed tissue to one location. In the present experiments we investigated the roles played by FGF supplied by co-transplanted 3T3 cells and by the temporary administration of synthetic steroids following cell transplantation. Treatments used for the different groups of animals are listed in Table 1. Group A received the same treatment as previously used for animals receiving transplanted clonal bovine adrenocortical cells (Thomas et al., 1997).

Fig. 1 shows survival data for the different groups of animals over 36 days following cell transplantation; at the end of this time surviving animals were sacrificed for assessment of histology and ultrastructure of the transplant tissue. As we previously demonstrated (Thomas et al., 1997), adrenalectomy is lethal in the strain of *scid* mice used in these experiments. Animals die 4–7 days after surgery (Fig. 1). The administration of synthetic steroid hormones (dexamethasone and fludrocortisone) allows animals to survive adrenalectomy, but when steroid administration is stopped, animals die within 5–8 days (Thomas et al., 1997).

Most animals that received transplants of primary bovine adrenocortical cells, using the variations in the protocol listed in Table 1, survived indefinitely. Omission of temporary synthetic steroid support over the first 7 days (groups B and E) resulted in some early deaths, although even in these groups most animals lived to 36 days.

In adrenalectomized animals bearing transplanted adrenocortical cells, the mouse glucocorticoid, corticosterone, is replaced by the bovine glucocorticoid, cortisol. Cortisol is not produced by the mouse adrenal cortex because the mouse adrenal does not express steroid 17α-hydroxylase, resulting both in the production of corticosterone rather than cortisol as the major glucocorticoid and also in the lack of adrenal androgen synthesis, which requires this enzyme (Perkins and Payne, 1988; van Weerden et al., 1992; Keeney et al., 1995).

All the adrenalectomized animals with transplanted cells that survived to 36 days had plasma cortisol at levels greater than the measured value in control nonadrenalectomized animals. In animals with transplanted cells corticosterone levels were low, but greater than those in adrenalectomized animals. Aldosterone was also present at levels substantially greater than those in adrenalectomized animals, but lower than those in control, nonadrenalectomized animals. Both aldosterone and corticosterone levels showed more variability than levels of cortisol.

Because the transplanted cells were derived from the zona fasciculata, we tested the possibility that the steroid in the mouse plasma that reacts with the aldosterone antibody was not in fact aldosterone. Specifically, the transplanted cells might secrete 18-oxocortisol, a steroid that can be synthesized from cortisol (Ulick et al., 1983) and that showed some

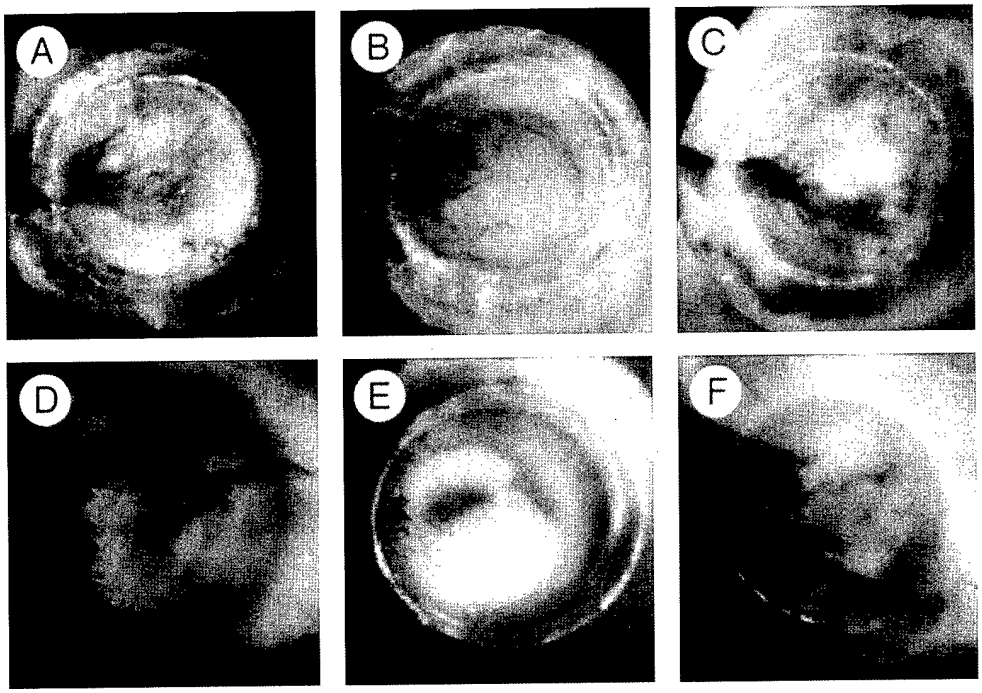

Fig. 5. Macroscopic appearance of the surface of the tissue formed from transplanted bovine adrenocortical cells in cylinders inserted under the kidney capsule, 36 days after cell transplantation. A through F show the appearance of the transplants in one animal from each group listed in Table 1. Magnification × 10.

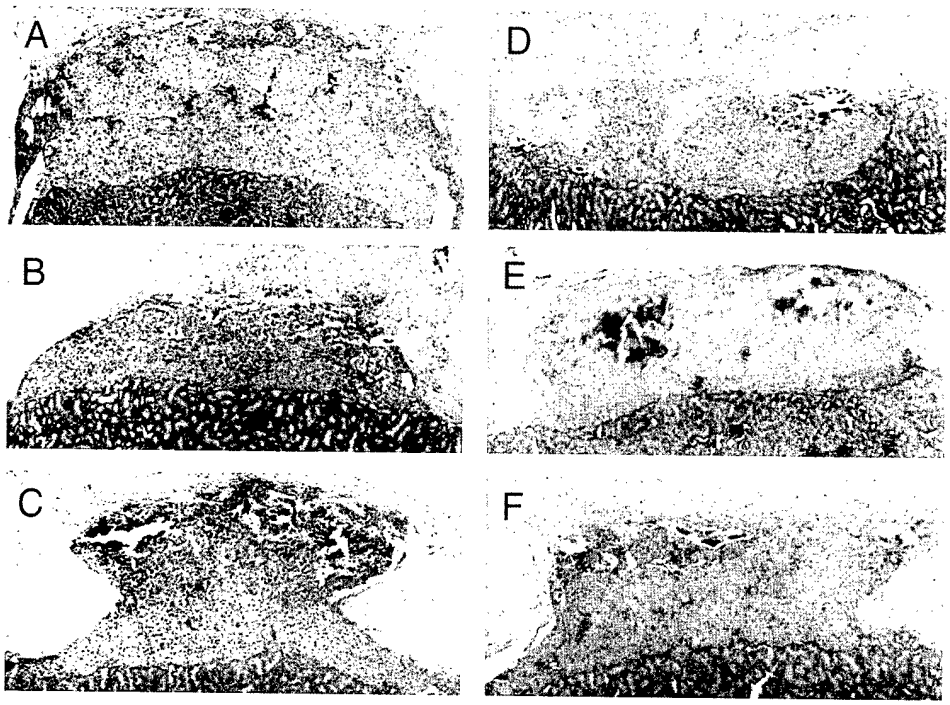

Fig. 6. Histology of tissue formed from transplanted adrenocortical cells, hematoxylin/eosin stain. Representative samples of the tissue formed from the cells in animal groups A through F (Table 1). Magnification × 40.

cross-reaction with the antibody we used. In order to test whether the steroid reacting with the aldosterone antibody required 17α-hydroxylation for its biosynthesis, we treated mice bearing transplanted adrenocortical cells with an inhibitor of 17α-hydroxylase, 22-amino-23,24-bisnorchol-5-en-3β-ol (22-ABC) (Ascoli et al., 1983; Sheets et al., 1985). 22-ABC potently inhibits 17α-hydroxylase activity in cultured bovine adrenocortical cells (Hornsby et al., 1986a). At the lower of two doses used, 22-ABC lowered cortisol levels without affecting aldosterone or corticosterone (Fig. 3). At the higher dose, the inhibitor completely suppressed plasma cortisol. At this dose, aldosterone levels were also somewhat lower, but corticosterone was similarly affected. The higher dose might inhibit other enzymes in the pathway of steroidogenesis, because although 22-ABC most potently inhibits 17α-hydroxylase, it can also inhibit other steroidogenic enzymes at higher concentrations (Ascoli et al., 1983; Sheets et al., 1985). These results indicate that 17α-hydroxylated steroids such as 18-oxocortisol do not contribute to the measured levels of aldosterone.

We investigated the time course of changes in plasma steroids in animals with transplanted cells and demonstrated that the steroids measured in plasma did in fact originate from the transplant tissue (Fig. 4). Following adrenalectomy, levels of all three steroids fell to undetectable levels. Measured values of cortisol in intact (nonadrenalectomized) animals probably do not represent authentic cortisol because the cross-reaction of the cortisol antibody with corticosterone would give the values observed; moreover, corticosterone levels in non-adrenalectomized animals are unaffected by the 17α-hydroxylase inhibitor 22-ABC (data not shown). Cortisol levels remained very low during the 7-day period of synthetic steroid supplementation. Following this period, cortisol, corticosterone and aldosterone levels were all higher, when measured at 14–36 days following cell transplantation (Fig. 4). To confirm that these steroids were produced only by tissue formed from the transplanted cells, the left kidney bearing the adrenocortical tissue was removed. Three animals in which nephrectomy was performed were allowed to survive a further 3 days, a period predicted to be less than the time to death resulting from deprivation of adrenal steroids. Plasma levels of steroids were very low at the time of sacrifice 3 days after nephrectomy (Fig. 4).

3.2. Histology and ultrastructure of tissue formed from transplanted cells

In those animals surviving for 36 days following cell transplantation, pale yellow tissue was visible within the cylinders, which had been invaded by prominent blood vessels from the capsule and surrounding connective tissue (Fig. 5). The tissue was fixed and processed for conventional histology or for electron microscopy.

The cylinder was carefully removed during processing, leaving the enclosed tissue formed from the transplanted cells intact and attached to the kidney. The

position occupied by the cylinder can be seen at the edges of the tissue in low-power views (Fig. 6).

In all groups the tissues were vascularized and had a histological appearance generally similar to that of the normal adrenal cortex. Tissues often showed adrenocortical cells arranged in cord-like structures, as in the zona fasciculata of the normal gland. Capillaries had large lumens, as in the normal gland. Staining with the DNA-binding dye DAPI confirmed that endothelial cells lining the capillaries were of mouse origin. Mouse cells may be distinguished from bovine cells by the presence of several brightly staining points in their nuclei (Thomas et al., 1997). Between the adrenal tissue and the kidney capsule was a variable amount of connective tissue containing only mouse cells. No connective tissue was observed where the tissue bordered the kidney (Fig. 7), which was typically separated by small blood vessels.

In groups A, B, C, and F, bovine adrenocortical cells were transplanted with FGF-secreting 3T3 cells. Tissues formed from adrenocortical cells transplanted with FGF-secreting 3T3 cells, both mitomycin C-treated and irradiated, had a uniform appearance; there was more variability in histological appearance of the tissues in the other groups. When 3T3 cells were omitted, the tissue formed in all animals had a necrotic region in the upper part of the tissue close to the kidney capsule, even though the rest of the tissue was apparently intact and healthy (Fig. 6). In the groups with 3T3 cells, this area of the transplant tissue typically contained many large blood vessels, presumably connecting to the vessels that enter the top of the tissue (Fig. 5). The necrotic area in the tissues without 3T3 cells occupied the region in which these vessels were observed in the other groups. However, when some animals without 3T3 cells, otherwise similar to those of group D, were allowed to survive for 70–100 days, rather then being sacrificed at day 36, the histological appearance of the tissue showed that the necrotic area had resolved and that large blood vessels were now present (Fig. 8).

When 3T3 cells were rendered incapable of cell division by irradiation (group F) the results resembled those obtained when the cells were rendered incapable of division by mitomycin C. If 3T3 cells were not treated to prevent cell division, they overgrew the transplant tissue, forming a hemangioma, as originally reported by Forough et al. (1993).

The proliferation rate of bovine adrenocortical cells in the transplant tissues at day 38 following transplantation was assessed by staining for the Ki-67 proliferation-associated protein (Fig. 9). Although we did not make precise measurements of the proliferation indices in the various groups, it was evident that the proliferation rate was much lower in all the tissues from animals that did not receive co-transplanted 3T3 cells.

3.3. *Ultrastructure of tissue formed from transplanted cells*

In general, the ultrastructure of tissue formed from transplanted adrenocortical cells closely resembled that of the normal adrenal cortex, and differed greatly from that of the cells in culture before transplantation. Mitochondria in transplanted cells usually had cristae in the tubular or vesicular forms characteristic of steroidogenic tissues (Neville and O'Hare, 1982) (Fig.

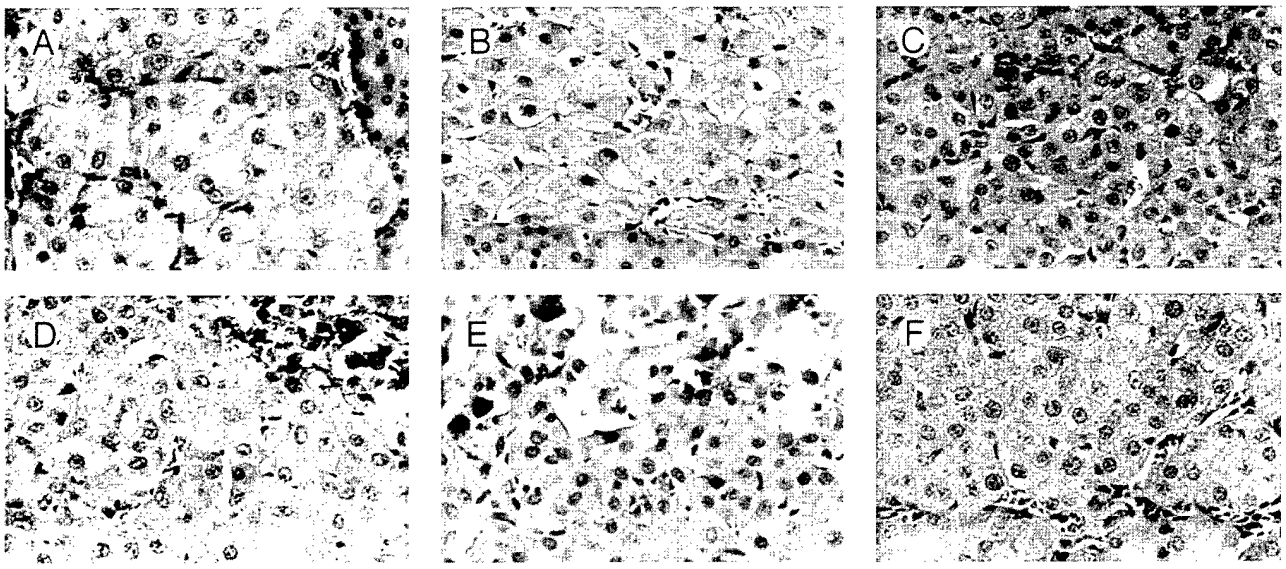

Fig. 7. Detailed histology of tissue formed from transplanted adrenocortical cells, hematoxylin/eosin stain. Representative samples of the tissue formed from the cells in animal groups A through F (see Table 1). The boundary of the adrenocortical tissue with the mouse kidney is visible in A, B, and F. D and E show samples of tissue adjacent to the necrotic areas seen in tissues in these groups. Magnification × 400.

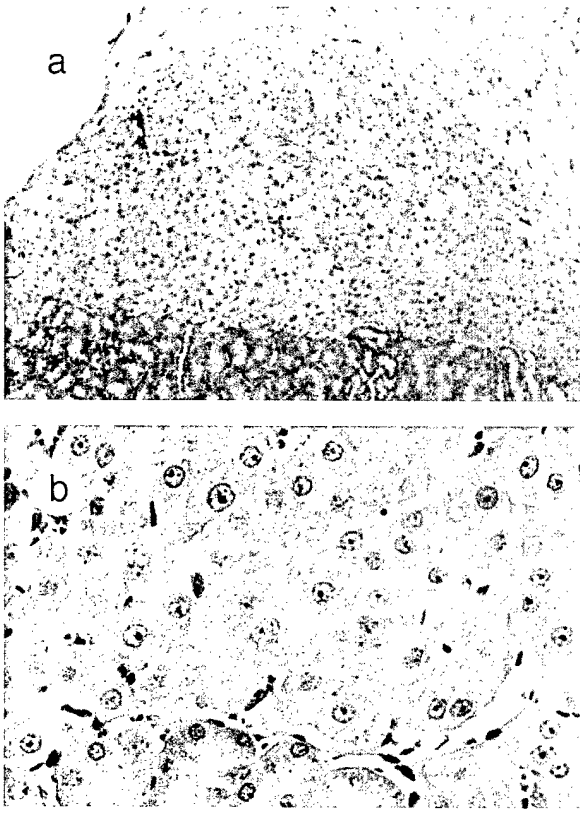

Fig. 8. Histological appearance of tissue of animals treated similarly to those in group D (Table 1) but allowed to survive to 70 days after cell transplantation. Hematoxylin and eosin stain. (a) Magnification × 40; (b) magnification × 400, showing boundary with the kidney.

10). In contrast, mitochondria in pretransplantation cells in culture showed only lamellar cristae. Whereas pretransplantation cells had large numbers of ribosomes and prominent rough ER, transplanted cells showed extensive development of smooth ER, less rough ER and fewer free ribosomes, features also characteristic of steroidogenic tissue (Neville and O'Hare, 1982) and also observed in the intact adrenal gland. Another prominent feature of the tissue formed from the transplanted cells was extensive contacts between cells consisting of many interdigitating microvilli (Friend and Gilula, 1972; Reaven et al., 1989; Plump et al., 1996). They were also observed in the bovine adrenal gland, but not in the cells in culture (Fig. 10).

The ultrastructure of the capillaries also resembled that of the normal adrenal cortex. Fenestrae were present in the mouse endothelial cells that have invaded the tissue derived from the transplanted cells; fenestrated endothelia are typical of endocrine tissues, and in the adrenal cortex increase in number in response to stimulation by adrenocorticotropic hormone (ACTH) (Idelman, 1970; Apkarian and Curtis, 1986).

4. Discussion

These experiments show that primary bovine adrenocortical cells transplanted into *scid* mice can reconstitute functional adrenocortical tissue that has many of the properties of the normal adrenal cortex. We previously noted that an important element for success of adrenocortical cell transplantation is the supply of FGF from co-transplanted FGF-secreting 3T3 cells (Thomas et al., 1997). FGF exerts potent effects on cell survival and apoptosis (Tilly et al., 1992), cell proliferation (Gospodarowicz et al., 1977), and angiogenesis (Forough et al., 1993). The 3T3 cells were rendered incapable of further division and were equally effective when this was accomplished by treatment with mitomycin C or by irradiation. At 36 days following cell transplantation, transplants that were not formed by the inclusion of FGF-secreting cells always contained a necrotic area, although the tissue produced plasma steroid levels in the same range as the 3T3 cell-containing transplants. Tissues formed with or without 3T3 cells, outside of the necrotic area, had very similar structures, indicating that the 3T3 cells do not substantially change the nature of the transplant tissue. Labeling studies indicate that the 3T3 cells locate within or next to the endothelium that forms within the transplant tissue (unpublished observations).

FGF from 3T3 cells appears to accelerate the formation of the mature transplant tissue structure. Without FGF-secreting 3T3 cells, the transplants eventually (by 70 days) achieve the mature structure observed in 3T3 cell-containing transplants at 36 days. By that time the necrotic area has resolved and large vessels are present in the upper part of the tissue, as observed in transplants with 3T3 cells. As a multifunctional protein, FGF could act via several different mechanisms to produce this result. First, because the proliferation rate of adrenocortical cells in transplants with 3T3 cells is higher (Fig. 8), it is possible that FGF acts as a direct mitogen for the transplanted cells, as it does in bovine adrenocortical cells in culture (Gospodarowicz et al., 1977). However, the Ki-67 labeling index is always much lower in transplants than in culture. In primary cultures, from which these cells were obtained for transplantation, most cells are dividing (Hornsby et al., 1992). If the proliferation rate of the cells in culture were maintained after transplantation, the transplant tissue would enlarge rapidly in a tumor-like fashion. In fact, all the transplant tissues observed here and in other experiments performed in this laboratory, even up to 300 days following transplantation, never exceeded the size observed here in the 'group A' animals at 36 days (Fig. 6). Similarly, plasma cortisol levels were always within the same range (Fig. 2). The apparently tight control of the function and size of the transplant tissue resembles that of the intact adrenal cortex in

vivo. We conclude that even if the primary effect of FGF from the 3T3 cells is on adrenocortical cell proliferation, this effect does not override the normal control of adrenocortical tissue size exerted by the hypothalamo–pituitary axis (Hornsby, 1985).

An alternate mode of action of FGF from co-transplanted 3T3 cells is on angiogenesis, because FGF is a potent angiogenic factor (Forough et al., 1993). In this model, FGF supplied by these cells acts by accelerating the production of a mature vascular system in the transplant tissue. Thus the increased rate of proliferation observed in the transplants with 3T3 cells at 36 days might be secondary to a greater extent of vascularization. However, angiogenesis clearly does occur in transplants that do not contain 3T3 cells. Although the control of angiogenesis in the adrenal cortex has not been investigated in detail, it is known that bovine adrenocortical cells have a high content of FGF-2, which is regulated by ACTH, and that human adrenocortical cells synthesize vascular endothelial growth factor (VEGF), also under ACTH regulation (Gospodarowicz et al., 1986; Schweigerer et al., 1987; Mesiano et al., 1991; Shifren et al., 1998). Presumably, in the absence of FGF from 3T3 cells, FGF or other factors required for angiogenesis are derived from the adrenocortical cells or from the host tissue, but are not present at levels sufficient to prevent the partial necrosis observed in the transplants at day 36.

Temporary steroid support of the adrenalectomized animals was not essential for the success of the transplants. In most animals, the function and structure of the transplants was not substantially affected by omission of the steroid supplementation, or by the provision of drinking water containing sodium chloride. However, in the absence of steroid supplementation, some animals succumbed to adrenal insufficiency in the first week after transplantation, presumably before the transplanted cells were able to secrete enough steroids to maintain the animals in a healthy condition. Additionally, nonsupplemented animals that survived beyond this period sometimes had lower or more variable levels of cortisol and aldosterone, or higher levels of corticosterone, than animals that had received steroid supplementation.

An interesting finding of the present experiments is that animals with transplants had readily measurable levels of aldosterone in the blood, albeit at a level less than that of nonadrenalectomized controls, even though the transplanted cells were derived from the zona fasciculata. This result contrasts with findings from the transplantation of clonal bovine adrenocortical cells. These clones were also derived from zona fasciculata cells (Hornsby et al., 1986b; Thomas et al., 1997). Within a group of animals that received transplanted cells of a single clone, most of the animals had cortisol in their plasma, but no aldosterone; however, occasional animals did have aldosterone, which was confirmed to originate from the transplant tissue (our unpublished observations). In animals with transplants that secrete aldosterone, it is possible that a subpopulation of transplanted cells might acquire glomerulosa-like properties following the formation of the cells into a tissue (Hornsby, 1985). Alternatively, bovine zona fasciculata cells might be capable of secreting aldosterone under certain circumstances (Chavarri et al., 1993). Although a separate enzyme, CYP11B2, is re-

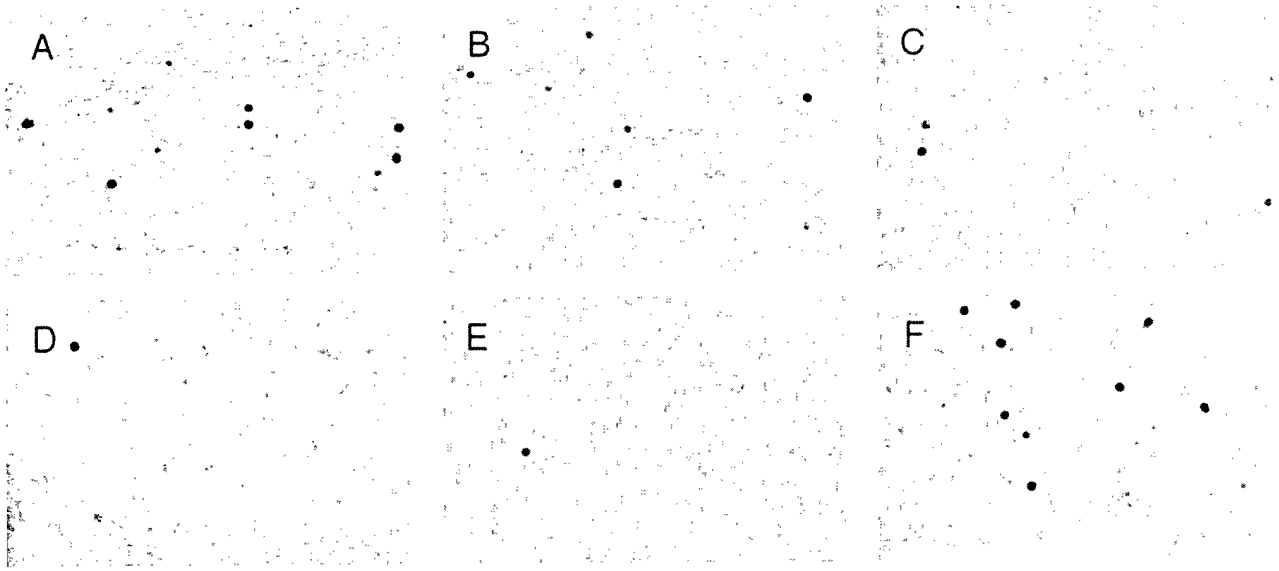

Fig. 9. Cell proliferation in tissues formed from primary bovine adrenocortical cells. Sections were stained with an antibody against the Ki-67 proliferation-associated antigen and were lightly counterstained with hematoxylin. Representative sections of the tissue formed from the cells in animal groups A through F (see Table 1). Magnification × 100.

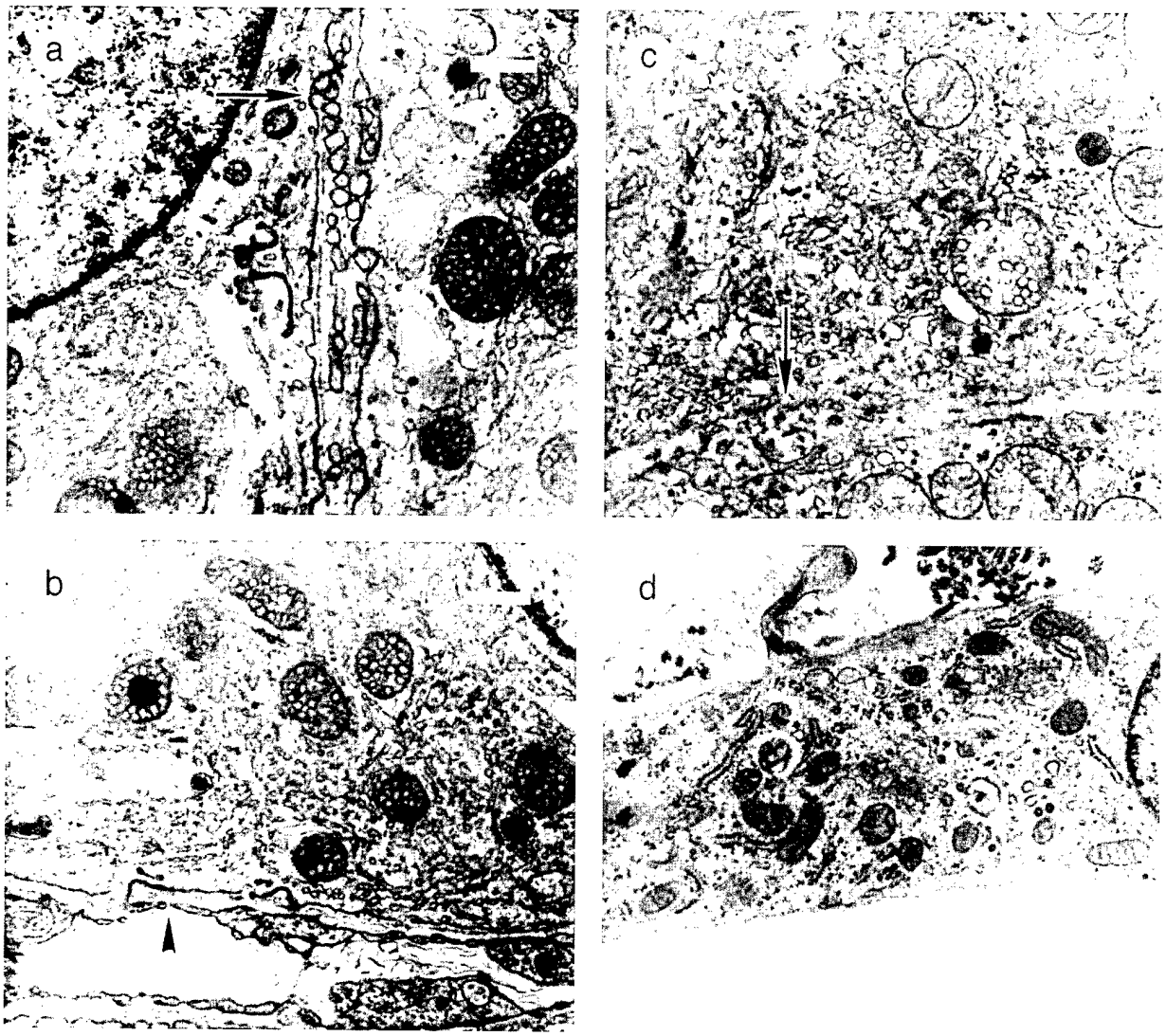

Fig. 10. Ultrastructure of tissue formed from adrenocortical cells. (a,b) Tissue formed from transplanted adrenocortical cells (group A); (c) normal bovine adrenal cortex; (d) primary bovine adrenocortical cells in culture. Large arrows indicate microvilli. Arrowhead shows fenestrae in the endothelium formed within the adrenocortical tissue. Scale bars, 500 nm.

sponsible for the conversion of deoxycorticosterone to aldosterone in other mammals, a specific member of the CYP11B gene family responsible for this conversion has not been described in cattle, and the mechanism of aldosterone biosynthesis in this species is still unresolved (Yanagibashi et al., 1986; Kirita et al., 1990).

In the present experiments, however, the possibility that glomerulosa cells were in fact transplanted together with fasciculata cells cannot be ruled out. The capsule of the adrenal gland with the attached zona glomerulosa is removed during the preparation of cells from the bovine adrenal gland (Hornsby and McAllister, 1991). However, it is not possible to avoid the inclusion of some glomerulosa cells in fasciculata cell preparations because, in the bovine adrenal cortex, the zona glomerulosa invaginates deeply into the zona fasciculata as sheaths surrounding blood vessels (medullary arteries) (Bachmann, 1954). These invaginations are clearly visible when in situ hybridization is performed with a zona fasciculata-specific probe, 17α-hydroxylase (our unpublished observations and ref. Didenko and Hornsby, 1996). On the other hand, aldosterone secretion is difficult to maintain in primary cultures of pure bovine glomerulosa cells, which acquire fasciculata-specific functions such as the expression of 17α-hydroxylase (Crivello et al., 1982; Crivello and Gill, 1983). Thus, even if glomerulosa cells were present in the initial primary cell suspension used here, there may have been a loss of glomerulosa-specific characteristics in the cells by the time they were transplanted. Therefore, it is not clear whether aldosterone secretion by the transplants originates from glomerulosa cells that were transplanted, or from fasciculata cells that change their phenotype in vivo to resemble glomerulosa

cells. Further experiments are required to distinguish between these possibilities.

Because levels of aldosterone achieved in animals with transplants were much lower than those of control nonadrenalectomized mice, and because we previously observed that animals bearing clonal adrenocortical cells without aldosterone secretion are nevertheless healthy, we cannot conclude that aldosterone is the essential steroid produced by the transplant tissue that rescues the animals from the normally lethal effects of adrenalectomy. Results from the clonal cell transplantation experiments suggest that cortisol or a cortisol precursor is able to substitute for the normal mouse adrenocortical steroids (Thomas et al., 1997).

In summary, the experiments reported here show that primary bovine adrenocortical cells transplanted into *scid* mice prevent adrenal insufficiency and form vascularized functional tissue. They document the relative importance of the supply of a growth factor, FGF, and of the provision of temporary steroid support in adrenalectomized animals receiving transplants of primary bovine adrenocortical cells. The transplantation procedure has been applied to clonal bovine adrenocortical cells and to primary human adrenocortical cells (unpublished observations and ref. Thomas et al., 1997). These systems may be used to investigate a variety of problems in adrenocortical cell biology, molecular biology, and physiology.

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DHEA: A Biologist's Perspective

Peter J. Hornsby, PhD

The recent extensive biomedical and lay public interest in dehydroepiandrosterone (DHEA) stems from the frequent suggestion that the marked age-related decline in the plasma level of dehydroepiandrosterone sulfate (DHEAS) in humans, discussed in this review, amounts to the development of a deficiency state for this hormone. Thus it has been proposed that replacement therapy with DHEA to restore youthful levels of DHEAS in older individuals might have beneficial effects on a variety of age-related conditions, such as cardiovascular and neoplastic diseases, diabetes, immune dysfunction, muscular weakness, and depression. Moreover, numerous effects of DHEA supplementation have, in fact, been documented; they are quite wide-ranging, and it is difficult to discern a single molecular mechanism that might account for all of them.

THE SECRETION OF DHEA BY THE ADRENAL GLANDS

DHEA and DHEAS (collectively designated DHEA(S)) are secreted in high amounts by the adrenal cortex only in humans and a few other primates. In adult humans, DHEAS circulates at very high concentrations (~10 μM), greatly exceeding the levels of other steroid hormones.[1,2] Plasma DHEAS levels reflect synthesis by the adrenal cortex because the adrenal is normally the source of almost all DHEA(S).[1] Small amounts of DHEA are also synthesized by the gonads. Because unconjugated DHEA is essentially an intermediate (to and from DHEAS), plasma levels of this steroid are not considered to be very significant,[3] in contrast to DHEAS, which is slower to change because of its very low metabolic clearance rate.[4–6]

Adrenal production of these steroids is negligible or absent in most laboratory and domestic animals, including rats, mice, guinea pigs, dogs, pigs, and cattle.[2] The adrenal glands in the adult mouse and rat lack the 17α-hydroxylase enzyme needed for the biosynthesis of DHEA.[7–9] Other species express the enzyme in their adrenal glands but do not make significant quantities of DHEA(S) for reasons that have been discussed elsewhere.[10]

In humans, the adrenal cortex has a specific layer, the zona reticularis, whose only known function is to secrete DHEA(S).[10,11] A morphological zona reticularis is present in nonprimate species but does not synthesize DHEA(S).[10] Although until recently the data were ambiguous, there is now direct evidence that the zona reticularis is the DHEA(S)-secreting zone and that the major zone of the cortex, the zona fasciculata, does not make DHEA(S)[11] (see Figure 1). This zone develops just prior to puberty at a time called adrenarche[1,13] (Figure 2). Before adrenarche, the cortex secretes cortisol, from the fasciculata, but very little DHEA(S). The nature of the factors that cause the development of the zona reticularis at adrenarche is unknown.[15–18] Adrenarche is observed only in species closely related to humans such as the gorilla and chimpanzee.[19,20] Primate species outside of this group, such as the rhesus and Cynomolgus monkeys, have measurable levels of DHEAS in plasma but do not undergo adrenarche and do not maintain the high adult levels of DHEAS found in humans.[2,21,22]

For editorial comment, see p 1402

The synthesis of DHEA(S) in adult humans and other primates presents several puzzles to the biologist. First, what is the function of DHEA in young adults? Equivalently, what is the driving evolutionary force that has caused high DHEA(S) production to be a feature of young adult life in humans? Second, why is DHEA(S) not synthesized in childhood before adrenarche? Third, why does the high rate of secretion in young adulthood show such a strong age-related decline? Fourth, is the age-related decline equivalent to deprivation of an essential hormone, and, therefore, is there a role for replacement therapy of DHEA in later life analogous to the use of estrogen-replacement therapy to prevent a variety of late-life diseases in women?

Because the human pattern of DHEA(S) biosynthesis (confined to adult life, commencing with the defined rise at adrenarche) is a recent evolutionary development, it is not possible to argue that in some way humans have inherited the biosynthesis of DHEA from ancestors in whom it had a function but that now that function has been lost. Simply from this argument alone, it seems likely that DHEA has an important function in adult life. High plasma levels of DHEAS in humans, at least under some set of circumstances, must have a beneficial effect on young adult survival to reproductive age or on reproductive success directly. Here, the available data on the modes of action of DHEA are reviewed, but it must be stated at the outset that the available data allow no firm conclusions as to what the essential function of DHEA might be. One should not overlook the possibility that its importance might be apparent only under the "wild" conditions under which humans evolved and that it might not have a critical function under present-day conditions.

AGE-RELATED DECLINE IN PLASMA DHEAS

The decline in plasma DHEAS from age 25 to age 70 (Figure 2) is one of the steepest continuous changes in the

Address correspondence to Peter J. Hornsby, PhD, Huffington Center on Aging, Baylor College of Medicine, 1 Baylor Plaza M320, Houston, TX 77030.

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DHEA — Brass Ring or Red Herring?

Gerontologists want so much to believe in DHEA. Early enthusiasm for DHEA – the celebrity steroid whose exploits are the focus of an impressive proportion of pop-science books on cures for aging and the proud possessor of hectares of shelf space in health food stores – seemed to be on fairly steady ground, at least in comparison with the scientific evidence for the antigeric properties attributed to melatonin and royal jelly. Epidemiological evidence suggested associations between low DHEA levels and high relative risks for breast cancer[1] and cardiovascular disease.[2] Studies of the metabolic effects of DHEA suggested that it might help to protect against the atherogenic effects of adult-onset diabetes and obesity.[3] A single injection of DHEA into old mice was said to correct many aspects of immunosenescence, including poor primary and secondary antibody responses to neoantigens.[4] Aging mice given high doses of DHEA in their food were lighter, had lower rates of kidney failure, and were cancer-resistant.[5] Besides, there was so much of it in young adults, and its levels declined so convincingly in older people, that it just had to do *something* good, didn't it?

New data and new arguments have begun to temper the excess of enthusiasm for this mysterious steroid. Follow-up studies have shown that high DHEA levels in mid-life do not, in the longer term, predict resistance to mortality from cardiovascular illness in men[6] or women.[7] Although high DHEA levels may be associated with resistance to breast cancer among premenopausal women, they seem to confer greater breast cancer risk in postmenopausal women.[8,9] High levels of DHEA do not seem to convey protection against cognitive failure[10] or obesity or glucose intolerance.[11] Thus the epidemiological evidence associating high levels of serum DHEA with resistance to the diseases and disabilities of old age is now tenuous at best.

Replications of the initial dramatic reports of immunorejuvenation in mice have been slow in coming, and interpretation of the rodent data has been complicated by the realization that rodents neither make DHEA nor retain ingested DHEA. Our own ongoing intervention trial of lifelong oral DHEA-S in genetically heterogeneous mice has, to date, shown no evidence for positive effects of this steroid on immunological responses or on survival (Miller et al., unpublished data). Similarly, clinical studies of DHEA as a potentiator of immune responses of middle-aged or older people to vaccination have been disappointing, providing little or no evidence for clinical efficacy in trials using influenza or tetanus vaccines.[12,13] DHEA administration to postmenopausal women for 3 weeks was found to have no positive effects on T cell proliferation or cytokine production, although the function of natural killer cells in the blood was increased significantly[14] in this double-blind cross-over study. A single-blind study in which DHEA was administered daily for 20 weeks to a group of older men (average age 64 years) produced no consistent or convincing changes in T cell responsiveness; the transient positive results claimed by these authors seem more likely to represent chance fluctuations over a long series of multiple comparisons,[15] and the study did not include parallel studies of placebo control subjects.

Long-term (6 to 12 month) administration of DHEA to volunteers has little or no effect on objective measures of physiological health.[15,16] Thus, a 6-month double-blind study of DHEA given at 50 mg/day to 30 volunteers aged 40 to 70 years found no effects on lipid profiles, insulin sensitivity, or body fat; the exception was a small decline in high density lipoprotein levels in women. A second study using DHEA at 100 mg/day for 12 months again found no change in lipid or apolipoprotein levels, lumbar muscle strength, glucose or insulin levels, or bone mineral density. Knee muscle strength increased in the men by about 15%, with the placebo effect responsible for half the change; knee strength decreased in the women. Androgen levels increased significantly among women in both of these studies, exceeding the normal range in the longer study. The only consistent dramatic effect was a psychological one: 67% of the men and 84% of the women in the 1-year study reported improvements in their own sense of well-being while ingesting DHEA.

See also p 1395

The current status of DHEA research suggests that learning more about the basic biology of steroid action should take precedence over the ever-alluring temptation to rush into clinical trials. Hornsby's review suggests a number of questions whose answers might help to point the way to a more judicious selection of physiological targets for intervention. The distribution among tissues of DHEA sulfatases, the ability of target tissues to transform DHEA into active metabolites, the source of and role for DHEA in the central nervous system, the interactions between DHEA and other steroids whose levels (and actions) may vary with age and gender, and the potential role of DHEA in peroxisome regulation — each of these areas is likely to reward additional investigation, and could help to guide a second, and possibly more productive, wave of clinical investigations.

Hornsby's review also provides an admirable example of the place for comparative biology in experimental aging research. One implication is fairly obvious: those of us who work in animal models need to remain watchful for exceptions to our tacit assumption that mice are simply small nocturnal people with prominent whiskers. Much of what we learn about the biochemistry, physiology, neurobiology, pathology, and genetics of mice, rats, dogs, and cats can be applied usefully, with appropriate caution and confirmation, to human biology, but much of it cannot. It is not simply that reports of DHEA administration to rats and mice will need to

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be re-labeled "pharmacology" rather than "hormone-replacement" studies, but rather that investigations of the action of this steroid in animals will need to take note of species-specific peculiarities in its uptake, transport, metabolism, excretion, and cellular actions, which may be very different in species that differ more than 100-fold in the natural levels of circulating DHEA. (The demonstration that nearly all common strains of laboratory mice lack the enzymes needed to synthesize melatonin[17] has led to a similar reassessment of the reported effects of pineal transplants and melatonin inhibitors in aging mice.)

The second implication is less discouraging: the restriction of DHEA to humans, chimps, and their close relatives, while arguing against an important general role of DHEA in mammalian aging, is consistent with the idea that this hormone may indeed play a key role in the timing of human life-history events. Evolution of long-lived primates has involved strong selection for genes that delay sexual maturation and for genes that postpone diseases to ages beyond those of full reproductive maturity. Physiological traits that sharply distinguish humans from their shorter-lived cousins – such as the unexplained predominance of DHEA among circulating human steroids, and its careful age-dependent regulation – may provide key clues as to how these antigeriatric tricks are accomplished.

Richard A. Miller
University of Michigan

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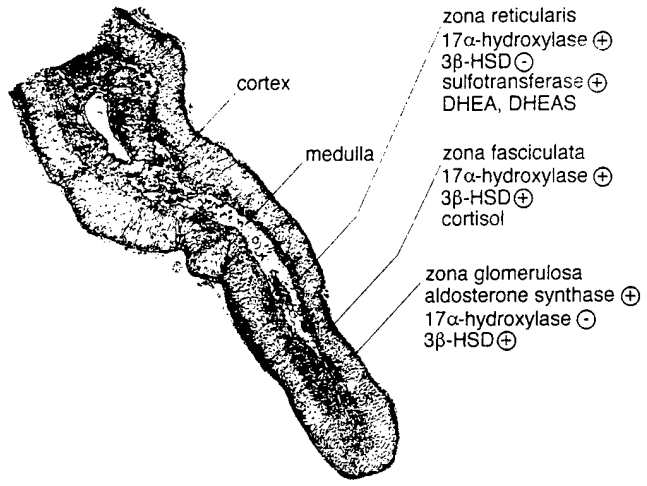

Figure 1. DHEA and DHEAS are secreted by a discrete layer of cells in the adult human adrenal cortex, the zona reticularis. The key molecular feature of the reticularis that results in the production of DHEA(S) is its low expression of the enzyme 3β-hydroxysteroid dehydrogenase, 3β-HSD.[11] The zona fasciculata has a high level of 3β-HSD resulting in the synthesis of the glucocorticoid, cortisol. Other key enzymatic differences between the zones that result in the production of different steroids by the zones are shown. (Adrenal gland section reproduced from ref. 12).

endocrine system in humans over this age range.[23,24] The long-term aim of research on the age-related decline is to account for the steep decline in adrenal biosynthesis of DHEA(S), to clarify the effects of aging per se versus the effects of ill health, and to establish whether the age-related decline has functional consequences. It shows a high inter-individual variability, caused, perhaps, by the heterogeneity of life histories in aging individuals.[25–27] The decline in DHEAS contrasts with the maintenance of plasma cortisol levels in aging.[28,29] Plasma cortisol levels rise to the same extent in young and old subjects after administration of ACTH or CRH, whereas the increase in DHEAS is much less in old subjects.[30,31]

The simplest hypothesis is that the decrease of DHEA(S) biosynthesis in aging results from a decrease in the number of functional zona reticularis cells (Figure 3). Unfortunately it is not possible to correlate the decline in individual DHEAS levels in aging with the morphological loss of the zona reticularis. In cross-sectional studies, there is a large, but variable, decline in DHEAS and variable changes in the zona reticularis. The hypothesis that loss of DHEA(S) biosynthesis results from loss of reticularis cells presumes that there is no feedback mechanism whereby levels of DHEAS regulate the width of the reticularis. Without a feedback mechanism, a loss of cells occurs without triggering a compensatory increase in the stimulus to DHEA(S) synthesis. Possible mechanisms for the age-related loss of reticularis cells have been discussed.[10]

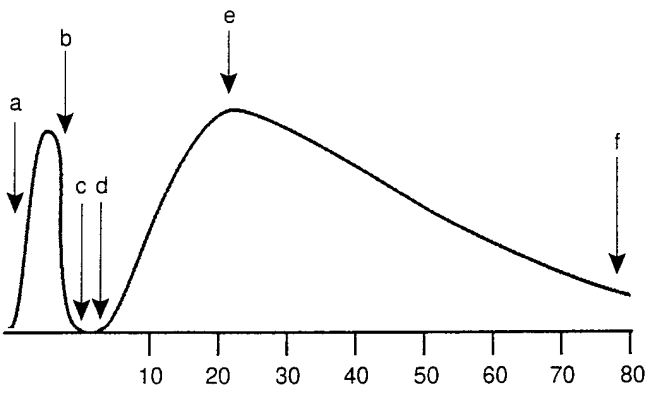

Figure 2. Diagrammatic representation of the plasma levels of DHEAS over the life span in humans. Before birth (a) the fetal zone of the adrenal cortex secretes large amounts of DHEA(S); following birth (b), the fetal zone rapidly involutes (c). The role of fetal DHEA(S) is outside the scope of this review (see ref. 14). At about age 6–7 years, the zona reticularis begins to develop in the adrenal cortex, and plasma DHEAS levels begin to rise (adrenarche) (d). The achievement of the peak level of plasma DHEAS in young adulthood (e) is followed by a progressive decline in adrenal secretion of DHEA(S), so that plasma DHEAS levels are often very low by age 70 and beyond (f).[1] The ordinate represents age in years; the scale is expanded before 10 years of age.

ACTION OF DHEA AS AN ANDROGEN

Although other modes of action are possible, the most likely is that DHEAS and DHEA serve as a source of active

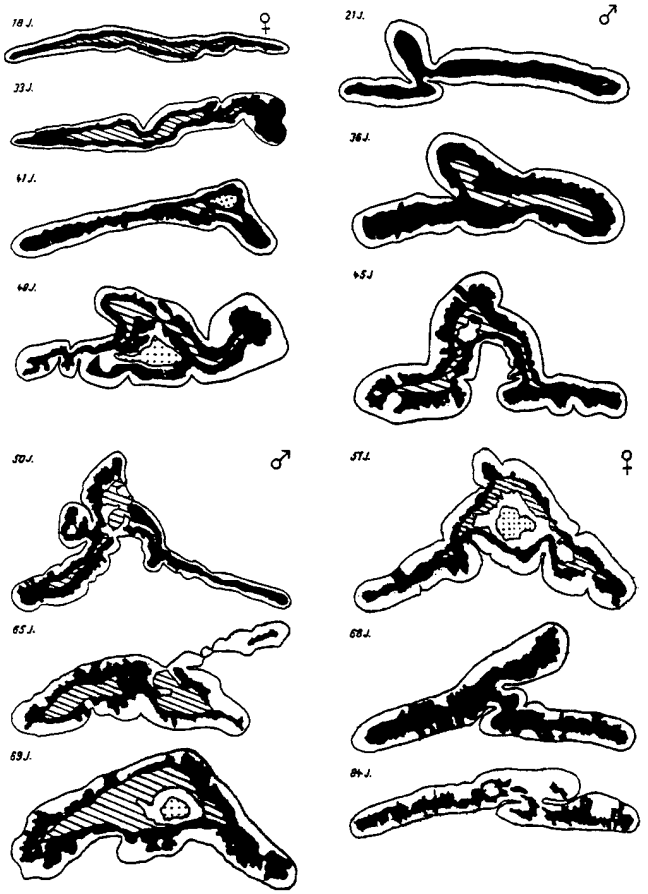

Figure 3. The age-related changes in the zona reticularis, hypothesized to result in a decrease in the number of functional reticularis cells. This figure, showing 12 adrenal glands (18 to 84 years), is reproduced from one of a very small number of studies of the changes in the reticularis with aging. It shows increasing irregularity rather than simple involution. Solid black, zona reticularis; white, zona fasciculata and zona glomerulosa; hatched, medulla; stipple, central vein.[32]

androgens in some subset of androgen-responsive tissues.[33] These steroids are often referred to as adrenal androgens, although this term is also used to refer to another steroid, androstenedione, which is also secreted by the adrenal cortex under some circumstances and which may be converted to testosterone in target tissues. However, androstenedione is secreted in large amounts only in genetic deficiency of 21-hydroxylase.[34] 21-Hydroxylase deficiency of the form termed "simple virilizing" results in masculinization in affected females at birth because the block of 21-hydroxylase leads to the accumulation of 17α-hydroxyprogesterone, which serves as a precursor for androstenedione.[34]

DHEA serves as a precursor for androstenedione in peripheral tissues, and excess DHEA(S) production by the adrenals also results in hyperandrogenization. The best evidence we have for this is that whereas the normal adrenal cortex in children secretes almost no DHEA(S), because of the lack of a developed zona reticularis, most malignant tumors of the adrenal cortex occurring in childhood, even though very rare, secrete DHEA(S).[35–37] The abnormal DHEA(S) production results in virilization, easily discernible in both males and females before puberty. Conversely, this androgenic effect provides a clear evolutionary rationale for why the secretion of DHEA(S) is suppressed during childhood, whereas it is prominent both before childhood (in fetal life) and subsequently in adult life. Excess DHEA(S) production in young women is also an established and relatively common cause of hyperandrogenism associated with problems that range from mild to severe (acne, hirsutism, infertility).[38–40] Many such patients may have "exaggerated adrenarche," hyperresponsiveness of DHEA(S) to ACTH.[41–43] Considering the available data, it is reasonable to call DHEA and DHEAS "adrenal androgens" provided one remembers that this is on the basis of their conversion to the more potent androgens, androstenedione, testosterone, and 5α-dihydrotestosterone (DHT).

Is the androgen effect the predominant reason the adrenal cortex secretes DHEA(S) during young adulthood? For clues to the answer to this question, one should look to the unusual circumstances under which the rise in DHEAS does not occur. Glucocorticoid replacement therapy for the treatment of congenital deficiency of 21-hydroxylase has, as a side effect, the suppression of DHEA(S) production by the adrenal glands, and such patients do not go through adrenarche.[44–46] However, puberty is not affected, and although many of these patients have problems resulting from incomplete control of androstenedione production by the adrenals, such patients have become pregnant and have given birth.[47] On the other hand, it is very hard to measure whether the overall fertility or health of such patients is affected by the absence of DHEA per se because such patients are under continuous medical treatment.

The timing of the rise of DHEA(S) production by the adrenal glands must surely provide the most significant clue to its function. The beginning of the rise just before puberty and the completion of the rise in young adulthood, together with the secretion of DHEA(S) in both sexes, suggests the possibility that DHEA plays a role in the final accelerated phase of linear growth (i.e., an anabolic function characteristic of androgens generally).[48] Possibly, the confinement of this pattern to humans and a few other primates is connected with the long maturational period of these species—few other mammals have a "childhood," a lengthy period of growth prior to sexual maturation. Under wild conditions, an effect of DHEA on maturation during young adulthood might give rise to increased fertility or increased reproductive success. In the absence of any good model organism or method to test this in humans, this concept has to remain purely theoretical.

INTRACRINOLOGY: DELIVERY OF ANDROGEN PRECURSOR TO TISSUES

An important idea, proposed by Labrie, is that DHEAS provides a universal precursor of further androgenic and estrogenic products that are produced in peripheral tissues as required to supply local needs.[49] This local production is regulated by the action of a series of DHEAS-metabolizing enzymes (steroid sulfatase, 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17β-hydroxysteroid dehydrogenase), yielding testosterone, which can then be acted on further by aromatase to produce 17β-estradiol or by 5α-reductase to produce DHT. All of these enzymes are widely expressed in peripheral tissues, but, importantly, aromatase is more restricted in distribution, with adipose tissue being a major site in humans but not in rodents.[50–52] Even the ovary may use circulating DHEAS as a precursor.[6,53,54]

The concept of intracrinology suggests that we should look for the function of DHEAS based on what each individual tissue does with this abundant precursor hormone. Labrie[49] comments that "It is remarkable that humans, in addition to possessing very sophisticated endocrine and paracrine systems, have largely vested sex steroid formation in peripheral tissues. While the ovaries and testes are the exclusive sources of androgens and estrogens in lower mammals, the situation is very different in higher primates, where active sex steroids are in a large part or wholly synthesized locally." Labrie estimates that 75% of estrogens to which target tissues such as the breast are exposed in young women originate from adrenal androgens and that this fraction rises to close to 100% after menopause. Thus, in the rat and guinea pig, castration reduces prostatic DHT to negligible levels, but in humans, castration reduces prostatic DHT only by 50 to 60% because of the supply of steroid precursor from DHEAS.[55]

Rodents and other species not only do not synthesize DHEA(S) in the adrenal gland, they also do not maintain high levels of plasma DHEAS when administered large doses of DHEA. Mice given access to 0.2 mg/mL DHEA in 20% ethanol consume ~1 mg per day (S.R. Northrup and P.J. Hornsby, unpublished observations). This is the equivalent of a daily oral intake of 3.5 g for a 70-kg human. However, in male mice the DHEAS level in plasma does not rise above the background for the radioimmunoassay; in female mice, the plasma level rises transiently to ~0.5 μM but then falls to negligible levels after a few days. The explanation for this behavior probably depends on an androgen-repressed steroid, sulfotransferase in mouse liver.[56] In male mice, the sulfotransferase is repressed so that little DHEA is sulfated. In female mice, the DHEA is transiently acted on by the sulfotransferase, producing a measurable level of DHEAS although much less than would be expected in humans based on the amount consumed.[57] Subsequently, however, the androgenic action of DHEA represses the sulfotransferase to the male level. Moreover, DHEAS appears to be excreted rapidly in rodents, as would be normally expected for a sulfate. The metabolic clearance rate is much lower in humans than in rodents,[4–6] implying a specific mechanism for renal reab-

sorption in humans as yet unexplored. Thus, although experiments in rodents on the effects of DHEA have been extremely valuable, we should always bear in mind the difficulty in applying rodent data to humans, where tissues have evolved to deal with the abundant precursor hormone by making their own sets of products, a situation that is unlikely to occur in rodents because they do not normally have high circulating levels of DHEAS.

In humans, on the other hand, the combined effects of secretion of the sulfate by the zona reticularis cell, the action of sulfotransferases, and possible reabsorption by the kidney all contribute to the high circulating level of DHEAS. Could the sulfate be the active form? There are two possible molecular modes of action of the sulfate per se. One is that DHEAS is an allosteric antagonist of the brain $GABA_A$ receptor (γ-aminobutyric acid/benzodiazepine receptor-chloride ionophore).[58] This role for DHEAS likely involves locally synthesized DHEAS, which is, in this case, a neurosteroid.[3,59] Because the binding and action of DHEAS on the $GABA_A$ receptor occur at concentrations that might exist in the brain, the action of DHEAS on this receptor may be physiological.[58] In humans, this action may be responsible for the effect of DHEA administration on REM sleep.[60] One possibility, therefore, for the role of the high level of DHEAS in human plasma is that it is intended to cross the blood-brain barrier (either as the sulfate or after conversion to the unconjugated form) and then act in the central nervous system. There is no reason to rule out this possibility, yet it makes little biological sense to flood the entire body with high concentrations of DHEAS simply so that some of it can enter the brain, although there is evidence that circulating DHEAS does directly or indirectly contribute to brain DHEAS.[59]

A second possible action of the sulfate is as an activator of the peroxisome proliferator-activated receptor type α (PPARα). In rodents, DHEAS induces a liver peroxisome proliferative response and is more potent than DHEA in this activity.[61] A metabolite of DHEAS appears to responsible because although DHEAS is inactive in a PPARα knockout mouse, it does not activate transfected PPARα in cultured cells.[61] PPARα is expressed in many tissues, functioning in the liver in the catabolism of fatty acids and xenobiotics and in the immune system as a factor in determining the duration of an inflammatory response.[62]

Despite these potential actions of DHEAS per se, it is clear that wherever there is sulfatase in peripheral tissues, DHEAS can serve as a local source of unconjugated DHEA. It seems inescapable that this is indeed the role for DHEAS and that, therefore, it is the sulfatase that is the key enzyme in the intracrinology of DHEA. The distribution of DHEAS sufatase activity in the rhesus monkey has been reported,[51] but this is not well established in humans. There is an X-linked steroid sulfatase in humans known to act on DHEAS, deficiency of which results in low maternal estrogens in a mother carrying an affected fetus and in ichthyosis after birth, but this is probably not the only sulfatase involved in the peripheral metabolism of DHEAS.[63]

DOCUMENTED EFFECTS IN HUMANS

How do we interpret the interesting effects of DHEA supplementation in older individuals? Far too little is known to allow firm conclusions (for an excellent recent review of this topic, see Miller and Schneider, ref. 63a). The number of controlled clinical trials that have involved DHEA is small (e.g., 3, 57, 64–68a, Ghusn, Baylor College of Medicine). The effects that have been documented in such trials, such as increases in levels or bioavailability of IGF-I, increased muscle strength, loss of fat and gain in lean body mass,[66–68a] are consistent with the activity of DHEA as an androgenic/anabolic agent. The complex interactions between insulin, plasma glucose, visceral fat, lipoproteins, cardiovascular disease, and DHEA and other androgens has been reviewed elsewhere.[69–73] Controlled studies have found immune system effects of DHEA, particularly in systemic lupus erythematosus.[65,74] Androgens generally have ameliorating effects in autoimmune conditions,[75] but the clinical results with DHEA, where the masculinizing effects in women were not considered serious, may result from intracrinology and a more selective action of DHEA than testosterone. The increased sense of well-being reported by subjects taking DHEA[66] is consistent with data from testosterone administration[76] but might also involve the neurosteroid activity of DHEAS. A major problem, from the point of view of a biologist trying to make sense of the varied reported effects of DHEA, is that clinical trials have not compared DHEA directly with testosterone; both steroids have been used in trials in older people, but separately, making comparisons difficult. (e.g., 77) The only sound conclusions on the actions of DHEA in humans will come from controlled clinical trials in both men and women and careful interpretation of their results.

POSSIBLE ROLE OF DHEA IN STEROID-DEPENDENT HUMAN CANCERS

Certain groups of women—European/American versus Asian/African and high versus low calorie intake—may be at increased risk of breast cancer because of higher amounts of body fat, particularly during adolescence and postadolescence, and thereby higher aromatase and local estrogen formation.[52, 78, 79] Estradiol, estrone, and androstenediol are active estrogens that act in tumor initiation and/or growth of small tumors; when tumors are larger they may remain estrogen-dependent or progress to estrogen-independence.[80] The role of androgens per se in breast cancer development and growth is unclear.[81] Depending on the level, androgens may affect breast cancer cells by occupying androgen receptors or by suppressing the effects of estrogens.[81] DHEAS may serve as an important source of both androgens and estrogens in the breast[33]; whether high DHEAS levels contribute to breast cancer is not clear. Prospective epidemiological studies in women have attempted to relate individual plasma DHEAS levels to subsequent breast cancer incidence.[33, 82, 83] However, these studies suffer from the problem that the true risk factor is likely the lifetime exposure of breast tissue to estrogens; single measurements of DHEAS may not be reliable enough to provide significant correlations with subsequent breast cancer development. DHEAS levels are depend strongly on the age of the patient at the time of sampling and on short-term influences, such as stress, and can vary considerably in an individual from month to month and year to year.[27, 84] Unconjugated DHEA levels are also variable over short time periods.[31] Moreover, in shorter term epidemiological studies, plasma DHEAS correlates with many parameters of physical and mental well-being.[85, 86] One example is that DHEAS levels decrease as HIV-positive subjects progress to AIDS, the decrease occurring as symptoms develop.[87,88] These findings suggest that poor health generally causes low DHEAS, as discussed in more detail elsewhere.[10]

Thus, what would be required would be to sample DHEAS levels repeatedly in young women during puberty and young adult life, followed by an assessment of lifetime risk of breast cancer. Exposure to estrogens might have effects on breast cancer development as early as fetal life,[88a] so only a lifetime study would be reliable, yet it is also clearly impractical. Although the question of the role of DHEA in induction of breast cancer is unresolved, it is clear that adrenal androgens can influence the growth of hormone-dependent tumors once they develop. The relevance of the adrenal as a source of precursors for androgens and estrogens was recognized in the earlier part of this century with the introduction of surgical and later chemical adrenalectomy as effective treatments for steroid-dependent breast and prostate cancer.[49, 89–91] Whereas the importance of the adrenal androgens in these diseases is still recognized, the high-risk procedure of adrenalectomy has been replaced by strategies designed to block local conversion of DHEAS to active androgens and estrogens.[49, 80, 92]

More information on the factors influencing human adrenocortical DHEA(S) production is needed to identify the hormonal or other factors that set the adrenal androgen production level. This information may define more precisely the risk factors of adolescent and postadolescent women for higher peak levels of DHEAS and consequent increased exposure of the breast tissue to adrenal-derived androgens and estrogens. This may allow appropriate intervention in high-risk women and may provide other avenues of rational treatment in estrogen-responsive breast cancer.

CONCLUSIONS

DHEA and its sulfate have or had an as yet unexplained importance in young adult life in humans, perhaps no longer critical under present-day conditions. The selective androgenic effect via intracrine conversion to more active steroids suggests a targeting to individual responsive tissues, but the degree of importance of this route versus the direct synthesis of androgens and estrogens by the gonads is not clear. Clinical data suggest that DHEA, like other androgens and estrogens, might have a role in hormone replacement therapy in older people, but as a potent steroid, with roles in the development and growth of hormone-dependent cancers, it should be used only in controlled clinical trials.

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The New Science and Medicine of Cell Transplantation

This novel approach to therapy also can provide basic information not accessible through other techniques

Peter J. Hornsby

Isolated human or animal cells may be transplanted directly into human patients or experimental animals. In their new hosts, these transplanted cells become a functional part of the body, either by integrating into an existing tissue or alternatively by forming a new tissue or organ in cooperation with host-derived connective tissue cells, blood vessels, and nerves.

It is only recently that cell transplantation has been recognized as a distinct discipline. It has emerged as a synthesis of several related areas of basic science and clinical therapy. First, it extends organ and tissue transplantation with the same clinical objectives as those classical techniques, i.e. the replacement of diseased or damaged organs and tissues and the correction of metabolic or hormonal defects. For example, much effort has gone into attempts to succesfully transplant pancreatic islet beta cells for the treatment of insulin-dependent diabetes.

A second contributing area has been ex vivo gene therapy. In this technique, transplantation of genetically modified cells (derived from the patient, or from another source) are transplanted as a vehicle for therapeutic gene and protein delivery, as an alternative to in vivo gene delivery by viruses or other means. Transplanted cells could be used to deliver a therapeutic protein to a site in the body where it is needed or to deliver it systemically into the circulation.

Third, the science and medicine of tissue engineering is closely related to cell transplantation, with the emphasis on combining cells with synthetic polymer matrices before transplantation. These biodegradable and biocompatible polymers serve to support the growth and function of the cells and act as a guide or scaffold for the newly formed tissue after transplantation. The first tissue engineering products are now commercially available, such as the Apligraf human skin equivalent.

As other techniques are developed, distinctions among these disciplines are likely to become increasingly blurred, as elements of each are combined in ever-more-sophisticated therapeutic regimes. For example, instead of replacing diseased tissue, future investigators may learn to stimulate the defective or damaged tissue, which might regenerate by using trophic factors secreted by a relatively small number of transplanted cells.

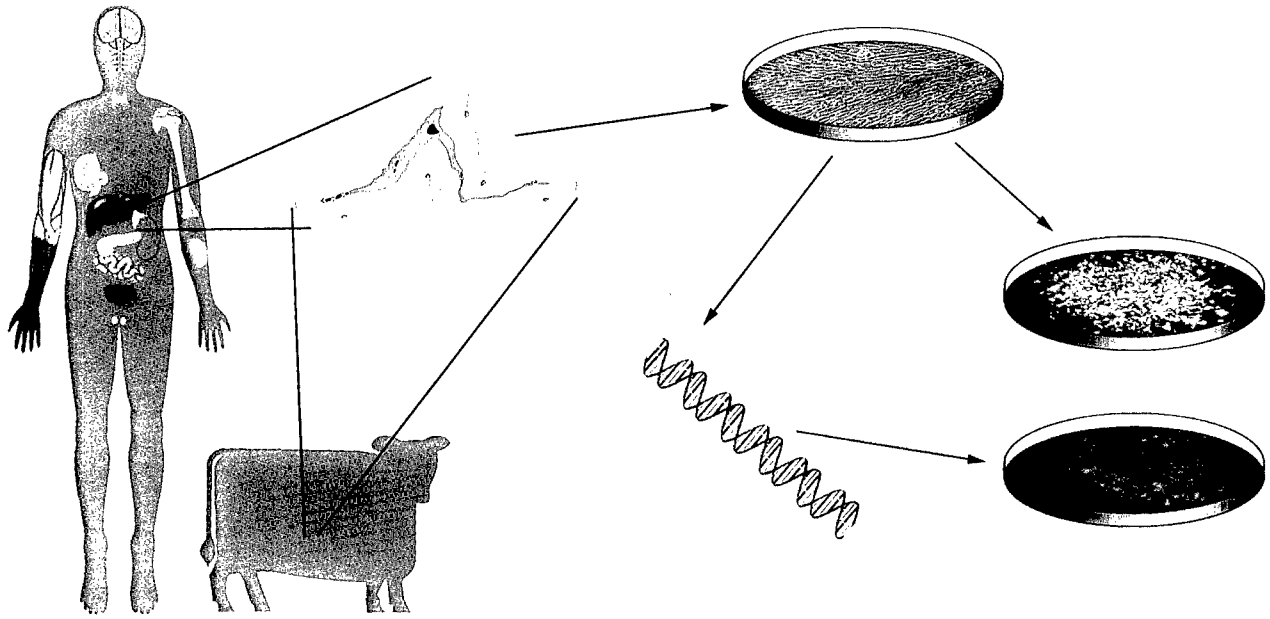
Despite the current emphasis on applying cell transplantation to solve medical problems, this technique also can be used to address basic science questions in physiology and biochemistry. Thus, by combining in vitro techniques, such as the growth of specialized cells in culture and genetic manipulation of cultured cells, with growth of cells in an appropriate host in vivo, investigators could construct tissues and organs from component cells and, in the process, learn more about cell origins, interactions, turnover, and senescence.

Transplantation of Cells of the Adrenal Cortex

Although several distinct cell types have been successfully transplanted in clinical procedures or in animal experiments, my colleagues and I are studying cells of the adrenal cortex, the part of the adrenal gland that synthesizes several steroid hormones, including cortisol, aldosterone, and androgen precursors (Fig. 1). These

Peter J. Hornsby is Associate Professor of Cell Biology at the Huffington Center on Aging, the Baylor College of Medicine, Houston, Tex.

FIGURE 1



On the left are shown various cell types that have been transplanted either in therapeutic procedures in humans or in experimental procedures in animals. These cells include neuronal precursors and other cells of the nervous system, myoblasts, bone marrow cells, chondrocytes, skin cells (keratinocytes and fibroblasts), blood vessel cells, thyroid cells, mammary epithelial cells, hepatocytes, pancreatic islet beta cells, urothelial cells, intestinal epithelial cells, and testicular Sertoli cells. In this article, the focus is on the transplantation of cells of the adrenal cortex. The figure illustrates the production of primary cultures of adrenocortical cells, from which both normal clones of cells and genetically modified clones may be derived (the latter illustrated here as cells expressing the gene for green fluorescent protein).

experiments entail transplanting human or bovine adrenocortical cells into mice lacking functional immune systems, because ordinary mice would otherwise promptly reject such transplanted cells.

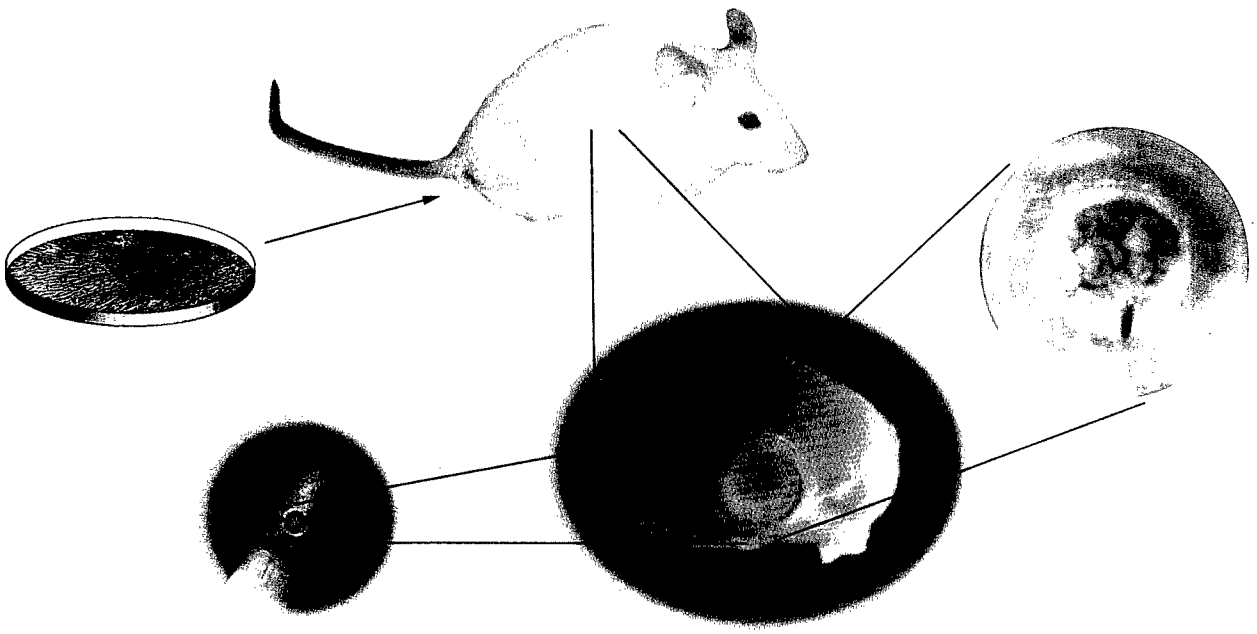
However, blood vessels in such immunodeficient animals readily establish connections with the transplanted cells. Ultimately, a structure resembling a miniature version of the normal adrenal gland is formed, and the cells secrete normal human adrenal steroids, which replace the adrenal hormones of the animals.

Our cell transplantation studies began as an outgrowth of studies of adrenocortical cells in culture. A variety of techniques enables us to isolate specific cell types and subsequently clone or genetically modify those cultured cells. However, cell culture systems do not provide the complete three-dimensional structure, cell-cell interactions, blood supply, extracellular matrix, and paracrine influences that cells have in vivo. Thus, although we can learn a great deal by studying such normal cells in culture, challenging them to form functional tissue structures in vivo can be viewed as a definitive test of normalcy, overcoming whatever happened to them in culture.

It is only recently that cell transplantation has been recognized as a distinct discipline

In our experiments we have adapted cell transplantation, tissue engineering, and ex vivo gene therapy to meet specific needs. For example, one typical scheme involves the implantation of a polymer material consisting of a polycarbonate cylinder (Fig. 2). However, in contrast to tissue engineers who have used other cell types, we find that adrenocortical cells do not require a polymer matrix to form transplant tissue. Thus, we use the polymer material only to form a chamber into which the cells may be injected. Adrenocortical cells appear to have an innate ability for self-organization, forming three-dimensional structures after aggregating from a dispersed cell suspension (Fig. 3).

FIGURE 2



Experimental transplantation of adrenocortical cells. The host is a mouse with the *scid* (severe combined immunodeficiency) mutation. In a single surgical procedure, the animal's own adrenal glands are removed and human or bovine adrenocortical cells are transplanted. To confine the cells within a defined space so that the growth, vascularization, and function of the cells may be more readily studied, we use a small polycarbonate cylinder that is inserted beneath the capsule of the kidney. The cylinder creates a space beneath the capsule into which the cells are introduced. When animals are killed 36 days later, pale yellow tissue is visible within the cylinder, which has been invaded by prominent blood vessels from the capsule and surrounding connective tissue.

Transplanted Adrenal Cortex Cells Are Fully Functional

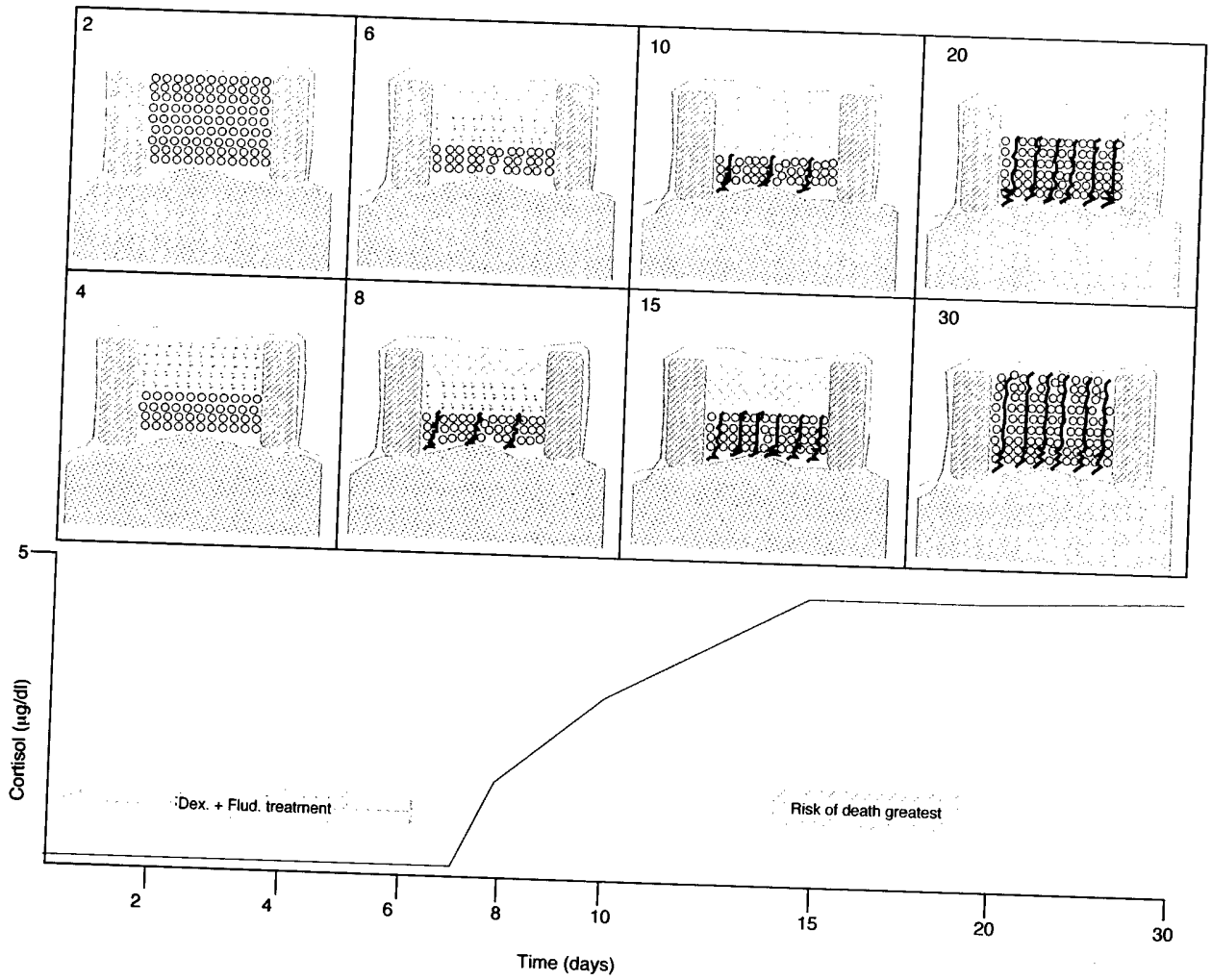
Both human and bovine adrenocortical cells form functional adrenocortical tissue after being transplanted. That tissue is fully vascularized and its ultrastructure and histology appear the same as that of the normal adrenal cortex. For both species, the steroids secreted by the transplanted cells rescue the animals from the typically lethal effects of adrenalectomy.

Moreover, there is no evidence of an immune reaction to the transplanted cells, which are a xenotransplant, consistent with the absence of B- and T-cell-mediated immunity in the immunodeficient mice. The tissues formed are chimeric, composed of human or bovine adrenal cells together with mouse cells (endothelial cells lining the capillaries, as well as fibroblasts and other cell types). Even after a year in vivo, the tissue is healthy and functional with no obvious evidence of cell death.

In the case of bovine cells, clones of cells derived from primary adrenocortical cells in culture also form adrenocortical tissue after transplantation. These experiments demonstrate for the first time that an endocrine tissue replacing a host animal's organ can be derived from a single normal somatic cell. In more recent experiments, we have genetically modified cells prior to transplantation. The transfected marker gene continues to be expressed in the tissue formed after the cells were transplanted.

One striking finding from our experiments is the difference in growth control in the cells before and after transplantation. In culture, cells are driven to rapid and continued growth by the conditions imposed on them. Their growth is determined by the culture medium, which contains fetal bovine serum and other growth factors, and by attachment to a flat substratum, which is required by most mammalian cells in vitro. When plated at low cell density, cells grow rapidly until they fill the available surface of the dish, slowing down when they become crowded and no longer have unlimited access to all components in the medium.

FIGURE 3



Scheme depicting events after transplantation of adrenocortical cells in a small cylinder introduced under the kidney capsule of *scid* mice. Numbers indicate days following cell transplantation. Initially there is some cell death (gray), but most cells in the layer next to the kidney survive (adrenocortical cells are purple, kidney tissue is orange). The cell debris is progressively replaced by mouse connective tissue (yellow). Capillaries lined with endothelial cells (blue) are observed in the viable adrenocortical tissue at early times. The tissue progressively fills the available space and achieves a functional vascularization united with that of the host. Note that the vessels supplying the transplant tissue mostly enter through the capsule and the connective tissue at the top of the transplant; this is not shown in this scheme. At the bottom, plasma cortisol levels are shown diagrammatically. The transplanted cells' synthesis of steroids, including cortisol, is suppressed over the first 7 days, when the animals are maintained with daily injections of the synthetic steroids dexamethasone and fludrocortisone. Cortisol synthesis begins following cessation of steroid supplementation. Death of the animal will occur at about 14 to 19 days if the transplant does not successfully take over its adrenal steroid requirements.

Growth Regulation In Vivo Differs from That In Vitro

Meanwhile, growth regulation in vivo is less a matter of regulation of cell division and more one of regulation of total tissue size. For endocrine tissues like the adrenal cortex, the body controls the total amount of functional tissue, thereby regulating overall levels of circulating adrenal hormones. The growth of adrenocortical cells in vivo appears to be regulated indirectly, mainly by control of angiogenesis, the growth of blood vessels in the tissue. The hormones that control the adrenal cortex, principally adrenocorticotropic hormone (ACTH), increase the local synthesis of angiogenic factors

such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF).

Successful cell transplantation requires that the host form new blood vessels, integrating the transplanted tissue into the host vascular system. This important process is not well understood. Transplanted adrenocortical cells support angiogenesis, the ingrowth of mouse endothelial cells, and the formation of a new vascular system. Yet this process differs from the unlimited angiogenesis that characterizes this process in cancer tissue.

Similarly, while adrenocortical cells proliferate in vivo in the transplant tissue, tissue growth is limited, approximating the size of the cylinder we used. Even after a year in vivo, the tissue is no bigger, yet it is healthy and maintains a constant level of hormonal steroids in the blood of recipient mice. In other words, it behaves as normal adrenocortical tissue and not like a malignant growth. Even though the tissue is a xenotransplant, the host vasculature and the transplanted cells cooperate to create a tissue that properly functions in the host animal over very long periods.

The Future of Cell Transplantation as Therapy

Harnessing cell transplantation methods for use in human therapy will involve constructing tissues to fulfill a wide variety of particular functions, using both normal and genetically modified cells. For example, a tissue could be constructed to secrete, under appropriate regulation, a therapeutic protein. In the case of insulin, cells should synthesize only the amount that is required, and the process should be responsive to blood glucose levels. Eventually, tissues could be constructed with structural and functional properties to replace damaged organs—perhaps even combining different cell types to construct organs to be transplanted.

Consider certain arguments aired during the recent debate over cloning of human beings, which began after investigators cloned sheep and mice. Some proponents of cloning said that it should not be prohibited because spare parts might be produced for transplantation procedures. However, the use of materials derived from human oocytes is not considered ethically acceptable by many people. On the other hand, deriving materials from somatic cells obtained from a patient's cells and manipulating them in vitro to form the tissues that are required does not appear to raise comparable ethical misgivings.

Producing transplant tissue from clonal cells—as is done in my lab for adrenocortical cell transplants—could make it possible to use cells that are normal but also homogeneous and genetically defined. Despite the cloning and genetic manipulation, the cells remain similar to the original cells; they are not genetically different except for the specific modification made. By freezing the clone in liquid nitrogen and subsequently expanding the thawed cells in culture in stages, sufficient cells of a single clone could be produced for thousands of transplantations. This approach is similar to that used with WI-38 human fibroblast cells that, although limited in proliferative potential, have been used in vaccine production for three decades.

Although the transplantation of adrenocortical cells could be used to correct adrenal steroid insufficiency, we also plan to use this approach for delivering therapeutic peptides. This approach differs from ex vivo gene therapy strategies in which genetically modified cells are injected as a dispersed population into a host organ. In such procedures, the cells do not form a discrete vascularized tissue. By contrast, a structure formed from adrenocortical cells could be implanted in a specific site and later removed from the host if necessary.

Although therapeutic cell transplantation procedures are currently viewed as high-tech and expensive, they may eventually lower health care costs. Surgical procedures needed for introducing such implants are considerably simpler than are conventional organ transplant procedures. If a diseased tissue or organ is replaced with healthy functioning tissue, particularly if derived from a patient's own cells, the reduced need for medical care may lead to savings in overall health care costs. In the current procedure, significant savings can be anticipated from improving efficiencies in cell culture and genetic modification procedures.

Transplantation To Study Physiology, Molecular and Cell Biology

Two features of cell transplantation make this method particularly suitable for studying basic human biology. One is that it uses a clonal cell

population, and the other is that it involves replacing an animal organ by transplanted cells from another source. Replacing animal cells with human cells produces a chimeric mouse/human model that can be used to address fundamental questions about human physiology and disease without conducting experiments on human subjects.

When genetically modified cells are used during transplant procedures, they become what may be termed a transgenic tissue. The power of germ line genetic modification in the mouse to answer important biological questions is well established. For human cells, genetic modification in cell culture has been similarly powerful in elucidating human gene function. However, although germ line modification of humans is not an acceptable option, studying transgenic tissues containing human cells within experimental animals is acceptable and could prove a useful means of studying how human genes function in such tissues in vivo.

Cell Transplantation for the Study and Therapy of Aging

Cell transplantation also could prove valuable for studying human aging and for providing therapies to counteract processes associated with aging. For example, human susceptibility to certain diseases increases strongly as a function of age. Changes in tissue structure and function in aging are an underlying cause of age-related diseases such as adult-onset diabetes, osteoporosis, Parkinson's disease, and Alzheimer's disease.

Many fundamental questions remain to be answered. For instance, to what extent do differences between the physiology of an old person and of that of a young person result from permanent age-related changes in the properties of the cells of the body? Cell transplantation can provide a powerful method for investigating such permanent cellular changes in aging.

Biologists have been debating for several decades whether "cellular senescence" or "replicative senescence" in human cells is part of the human aging process. Normal human cells show a limited proliferative potential when grown in culture, a phenomenon first carefully described by Leonard Hayflick in 1961 that now often is called the Hayflick limit.

In simple terms, when human cells divide, the absence of telomerase activity in somatic (nongerm line) cells causes telomeres to shorten progressively. After this process has occurred for some 50 cell generations, the short telomeres are interpreted by the cell as a damage signal, which triggers the operation of a set of "senescence genes." This is similar to the response of cells to DNA damage that occurs as part of the mechanism by which human cells are protected from cancerous changes. If cells do not traverse the pathway of events that leads to permanent growth arrest (replicative senescence), they alternately may enter a pathway that leads to cell death (apoptosis). In either case, the cell carrying a damaged genome is prevented from future replication.

Researchers have cloned the gene encoding the catalytic component of human telomerase. When expressed in senescent human cells, this gene extends the replicative potential of those cells in culture. Some biologists now speculate that engineering other human cells to express telomerase would help to make them available for use in cell transplantation therapies. However, cellular aging encompasses a variety of changes in cell function, not just replicative senescence.

Cell transplantation could prove valuable for studying human aging and for providing therapies to counteract processes associated with aging

In my lab, we are investigating how aging affects the ability of human adrenocortical cells to form a functional, vascularized adrenal tissue structure after transplantation into immunocompromised, SCID mice. Aging incorporates processes that occur when cells grow for extended periods in culture and those processes that occur in the body over long periods, which we study by obtaining cells from donors of different ages.

By transplanting cells into a neutral environment—a "level playing field," as it were—we can test how cellular behavior and patterns of gene expression change with aging. This is most difficult to study directly in cells in animals as they age, because many molecular changes in aging, involving differences in the levels of indi-

vidual proteins, mRNAs, and lipids, are secondary to changes occurring elsewhere in the body. Notably, changes in circulating hormones have profound effects on the levels of molecules in their target cells. In other cases, changes in molecules are secondary to changes in cell populations, such as increases in connective tissue or fat within an organ. These effects are removed when "old" cells are placed into a "young" host environment.

Cell transplantation experiments can help elucidate both universal cellular aging processes, the changes that affect all cells in aging, and those unique aging processes that occur in specific cell types. In the future, as we acquire a more complete understanding of these cellular aging processes, this knowledge could be applied as part of cell transplantation therapy.

For instance, cells from a patient could be grown in culture, and age-related defects in the cells could be corrected by appropriate genetic manipulations. After expansion of a specific cell population, the rejuvenated cells could be inserted into the patient. Although this scheme is now science fiction, it might become conventional medicine during the 21st century.

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Subcutaneous transplantation of bovine and human adrenocortical cells in collagen gel in *scid* mice

Nikolay K. Popnikolov and Peter J. Hornsby
Huffington Center on Aging and Department of Cell Biology
Baylor College of Medicine
Houston, TX 77030
USA

For Correspondence:

Peter J. Hornsby, Ph.D.
Huffington Center on Aging
Baylor College of Medicine
1 Baylor Plaza M320
Houston, TX 77030
Tel: 713-798-7334
Fax: 713-798-6688
E-mail: phornsby@bcm.tmc.edu

Running head: Transplantation of adrenocortical cells in collagen

Abstract

Adrenocortical cells of bovine origin and of adult and fetal human origin were transplanted subcutaneously in *scid* mice after being embedded in collagen gel. In this site the cells survived, became vascularized by invasion of host endothelial cells, and secreted steroids into the circulation. The animals' own adrenal glands were removed at the time of cell transplantation. Steroids secreted by the transplants replaced the essential functions of the animals' own adrenal glands. Adrenalectomized animals without transplanted cells died after several days, but most animals with transplanted bovine or adult human adrenocortical cells survived; fewer animals survived with transplanted fetal human adrenocortical cells. The histology of the tissues formed from transplanted cells resembled that of the normal adrenal cortex. A few proliferating cells were observed in tissue from bovine or adult human cells; there was a greater percentage of dividing cells in tissue derived from fetal cells. Subcutaneous transplantation of bovine or human primary adrenocortical cells in collagen provides a model for the study of the physiology, cell biology, and molecular biology of adrenocortical cells in a three-dimensional vascularized tissue structure in a host animal.

Keywords: Adrenocortical cells, collagen, proliferation, vascularization

Introduction

A powerful use of cell transplantation is the study of basic aspects of the biology, physiology, and aging of cells from species in which *in vivo* experimental manipulations are not feasible, such as large animal species and, in particular, humans. This basic role of cell transplantation complements its roles in correction of metabolic defects, *ex vivo* gene therapy, and tissue engineering. Transplantation of adrenocortical cells has potential applications both in basic science studies and in cell therapy (1). Adult rat adrenocortical cells have been successfully transplanted beneath the capsule of the kidney (2, 3) and fetal rat adrenocortical cells have been transplanted subcutaneously (4). Previously, work from this laboratory showed that primary and clonal bovine adrenocortical cells may be transplanted under the capsule of the *scid* mouse kidney. There they survive and form a vascularized tissue which can replace the essential functions of the animals' own adrenal glands (5 - 7). The animals are adrenalectomized in the same surgical procedure used for cell transplantation. Unless the transplanted cells secrete adrenocortical steroids the animals will die of adrenocortical insufficiency. Thus long-term survival of the animals is a marker for the success of the cell transplant. Additionally, because bovine and human adrenocortical cells secrete cortisol rather than corticosterone, the major glucocorticoid in mice, the replacement of corticosterone in plasma by cortisol also indicates the success of the transplant (5 - 7).

In preliminary experiments we had tested whether bovine adrenocortical cells would survive when transplanted subcutaneously, but they failed to produce functional tissue in this site (6). The success of the subcutaneous transplantation of human mammary epithelial cells in collagen gel (8 - 10) encouraged us to re-investigate the possibility of transplanting adrenocortical cells subcutaneously rather than in the kidney. The site is surgically easier to use, is not restricted with respect to the number of cells that may be transplanted, and allows ready access for further manipulation or intervention in the transplant tissue, when that is required. In the present experiments we show that functional tissue, closely resembling that of the normal adrenal cortex, can be formed by normal human and bovine adrenocortical cells when transplanted subcutaneously in *scid* mice when embedded in a matrix of type I collagen.

Methods

Growth of bovine and human adrenocortical cells in culture

Bovine adrenocortical cells were derived by enzymatic and mechanical dispersion of adrenocortical tissue as previously described (11, 12). Human adrenocortical cells were prepared from tissue from adult organ donors (2 males, ages 40 and 44), or alternatively from the definitive zone of fetal glands (16 to 20 weeks gestation) (13). Protocols for the acquisition of human adrenocortical tissue were approved by the institutional review board of Baylor College of Medicine. Primary cell suspensions were stored frozen in liquid nitrogen. Frozen cells were thawed and replated in Dulbecco's Eagle's Medium/Ham's F-12 1:1 with 10% fetal bovine serum, 10% horse serum and 0.1 ng/ml recombinant FGF-2 (Mallinckrodt, St. Louis, MO) (11, 12). Cells were grown in culture for 7 days before transplantation.

FGF-transfected 3T3 cells

A line of 3T3 cells stably expressing FGF-1 fused in frame with a signal peptide from hst/KS3, yielding a highly angiogenic secreted product (14), was generously supplied by T. Maciag. 3T3 cells were grown under the same conditions as bovine adrenocortical cells. To render the cells incapable of further division after transplantation, they were treated with mitomycin C (5) Cells were incubated at ~20% confluence for 24 hours with 2 μg/ml mitomycin C (Sigma, St. Louis, MO).

Preparation of collagen gel and adrenocortical cells for transplantation

Collagen was prepared as described previously (15, 16). Rat tail collagen fibers (3.0 g) were sterilized in 70% alcohol and dissolved in 500 ml 17 mM acetic acid. The mixture was centrifugated at 10,000 g for 1 hour at 4 °C and the supernatant was used as a stock solution. Before use, the ice-cold collagen solution was neutralized and adjusted to the proper osmolarity by adding a mixture of NaOH and 10x Hank's Balanced Salts Solution containing 200 mM HEPES. For each batch of collagen, different dilutions were tested. A dilution was chosen that gelled in approximately 10 minutes at room temperature.

The bovine or human adrenocortical cells were mixed first with mitomycin C-treated 3T3 cells at a 1:5 ratio. Cells were released from the culture dish by digestion with bacterial protease, aliquotted and pelletted by centrifugation (11, 12). Disposable 1-

ml syringes to be used for cell transplantation were prepared by cutting off the tip and the end of the barrel of the syringe. 100 - 150 μl collagen solution, into which had been mixed 3 to 5 x 10^6 cells, was pipetted into the modified syringes and allowed to gel at room temperature.

Subcutaneous transplantation of cells in collagen gel in scid *mice*

ICR *scid* mice were maintained in an animal barrier facility as a breeding colony. Animals were housed in a temperature-controlled room with a fixed 12-h light/dark cycle. Animals (both males and females) at an age greater than 6 weeks (~25 g body weight) were used in these experiments. Procedures were approved by the institutional animal care committee and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

In some experiments animals received the transplanted cells and were adrenalectomized in the same surgical procedure. However, it was convenient to perform the adrenalectomy on the day before transplantation, because this enabled the preparation of collagen gels containing cells that could be injected into a group of animals within a short time, avoiding the necessity for extensive storage of the cells/collagen gel mixture.

Tribromoethanol anesthesia was used for the procedures of adrenalectomy and cell transplantation. The surgical procedures used, including bilateral adrenalectomy and postoperative care of the animals, were modified from those employed in subrenal capsule cell transplantation (7). The midline skin incision used for adrenalectomy was also used for cell transplantation. Collagen gels containing adrenocortical cells were implanted subcutaneously near mammary glands numbers 2, 3 and 4 in a pocket between the skin and the underlying tissues. The skin was retracted to gain access to the site of transplantation and a pocket of ~1 cm in length and ~3 mm in width was created with fine blunt forceps. The gel containing adrenocortical cells was gently extruded from the end of a modified syringe into the pocket. The skin was closed with surgical staples.

Blood samples for assessment of circulating adrenal steroid levels were taken at weekly intervals. Samples were taken in the afternoon, 15 minutes after the injection of ACTH (Sigma, 0.01 units per gram body weight). Radioimmunoassays for cortisol, aldosterone, and corticosterone were performed as described (7).

Histology and immunohistochemistry

The fixation, paraffin embedding and histological examination of tissue formed from transplanted cells were carried out using standard techniques. Some tissue sections were stained for expression of the Ki-67 proliferation-associated antigen using monoclonal antibody MIB-1 (Immunotech, Westbrook, ME) and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA), as recommended by the manufacturers. This antibody does not recognize the mouse homologue of Ki-67 but recognizes the bovine protein with staining equivalent to that observed with human tissues (our unpublished observations). Sections were lightly counterstained with hematoxylin.

Endothelial cells were visualized by incubation with biotinylated *Griffonia simplicifolia* lectin I (10 mg/ml, Sigma) followed by incubation with avidin-biotin-peroxidase complex as for immunohistochemistry.

In situ hybridization

To distinguish cells of host origin from the transplanted cells in the tissue, species-specific satellite DNA probes were used for *in situ* hybridization. Human alpha satellite probe DNA and mouse gamma satellite probe DNA were synthesized by the polymerase chain reaction (PCR) using human and mouse genomic DNA as template. The primers used were (for human) WA1 (5′-GAAGCTTA(A/T)(C/G)T(C/A)ACAGAGTT(G/T)AA-3′) and WA2 (5′-GCTGCAGATC(A/C)C(A/C)AAG(A/T/C)AAGTTTC-3′) and (for mouse) WGS1 (5′-CCCAAGCTTGAAATGTCCACT-3′) and WGS2 (5′-CCCAAGCTTTTTTCTTGCCA TA-3′) (17). Bovine satellite I DNA was prepared as previously described (18). Species-specific satellite DNAs were labeled with digoxigenin-11-dUTP (Boehringer Mannheim Corp., Indianapolis IN) using the random primer method.

Deparaffinized 5 μm sections were dehydrated through ethanol and vacuum-dried. The sections were then treated with 0.2 N HCl for 20 minutes at room temperature, rinsed 3 times in water, and digested in 20 μg/ml proteinase K (Sigma) for 25 minutes at 37 °C. The sections were rinsed twice for 5 minutes in 0.2% glycine/PBS and PBS. The probe was added to the hybridization solution (55% formamide, 2 x SSC, 10% dextran sulfate, 2x Denhardt's solution, 0.1% Triton X-100, and 0.5 mg/ml herring sperm DNA) to a final concentration of 5 ng/ml. The probe solution was denaturated at 95 °C, chilled on

ice, added to the slides and sealed with a coverslip. The slides were placed into a preheated humidied chamber at 92 °C for 10 minutes, and then cooled for 10 minutes at -20 °C. Hybridization was performed at 37 °C for 16 hours. The slides were washed for 30 minutes each in 1 x SSC and 0.1 x SSC at 37 °C. The bound probes were visualized by incubation with a 1:250 dilution of alkaline phosphatase-sheep anti-digoxigenin Fab fragment (Boehringer Mannheim) followed by substrates (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium). Sections were photographed without counterstaining.

Electron microscopy

Tissue formed from the transplanted cells was fixed with 2.5% glutaraldehyde in PBS, post-fixed in osmium tetroxide, and embedded in Epon. Sections (100 nm thick) were stained with uranyl acetate and lead citrate and were examined and photographed by electron microscopy.

Results

Transplantation of bovine and human adrenocortical cells in collagen gel

Primary bovine or human adrenocortical cells, after a brief period in cell culture, were transplanted into *scid* mice in collagen gel using a subcutaneous site adjacent to a mammary fat pad. Successful cell transplantation was defined as the production of tissue from the adrenocortical cells at the site of transplantation, associated with healthy survival of the animals. We confirmed the lethality of removal of the adrenal glands when adrenocortical steroids were not replaced; in a group of 8 animals, 7 died by 10 days following surgery. Animals receiving successful adrenocortical cell transplants survived beyond 20 days following transplantation; no deaths occurred beyond 20 days. Additionally, success of transplants was observed by the appearance of cortisol in the blood, replacing the mouse glucocorticoid, corticosterone, which declines because the mice are adrenalectomized. Both cortisol and aldosterone were undectable in adrenalectomized mice that did not receive cell transplants. These results are summarized in Figure 1.

Macroscopic appearance of tissue formed from the transplanted cells

Figure 2 shows an example of a well-developed nodule of adrenocortical tissue formed from transplanted bovine adrenocortical cells. Typically we found a red or

yellowish nodule at the site of transplantation which showed invasion by blood vessels from surrounding connective tissue or fat. Although a single, more or less spherical nodule was often recovered, the tissue was also often found to be present as several smaller nodules, which were interconnected or completely separate.

Histology and histochemistry

In most experiments, adrenocortical cells were co-transplanted with FGF-secreting 3T3 cells, as previously described for subrenal capsule transplantation (5, 7). These cells were rendered incapable of division by mitomycin C treatment. We included these cells with the transplanted adrenocortical cells because we hypothesized that a critical step in their long-term survival and function would be their ability to become vascularized. Therefore, to promote angiogenesis in the transplanted cells, we supplied a source of a potent angiogenic factor, FGF (14). The results from subcutaneous transplantation in collagen gel resembled data previously obtained with transplantation into the kidney; when FGF-secreting 3T3 cells were omitted, the transplanted cells survived and functioned, but the tissue formed was smaller and often showed a core of tissue that appeared to be dying (Figure 3).

Detailed microscopic examination of nodules of bovine adrenocortical tissue showed many areas with the characteristic histological appearance of the adrenal cortex (Figure 4). Cells were often arranged in cord-like structures typical of the zona fasciculata. There was extensive vascularization. The capillaries surrounding the cords of adrenocortical cells could be clearly visualized by staining with *Griffonia* lectin (Figure 4b). Cell proliferation was observed as evidenced by immunohistochemical demonstration of Ki-67 antigen (Figure 4c).

The size and shape of nodules formed from transplanted adult human adrenocortical cells was more variable than that from bovine adrenocortical cells. In some cases large nodules were formed (Figure 5). These nodules showed cells in cord-like structures surrounded by large sinusoidal capillaries. Very few proliferating cells were observed when sections were stained for Ki-67 antigen (Figure 5b). Transplantation of fetal human definitive cells produced less extensively developed tissue without the sinusoidal capillaries (Figure 5c). Proliferation of these cells, however, was readily observed (Figure 5d).

These observations indicated that bovine adrenocortical cells, adult human adrenocortical cells, and fetal human adrenocortical cells all become invaded by host endothelial cells but that the patterns of vascularization differed among the tissues formed. Because very few or no endothelial cells are present in the cell cultures used for transplantation (19, 20), the endothelial cells in the transplant tissues appeared to be of host mouse origin. We confirmed this by *in situ* hybridization using satellite DNA probes specific for bovine, human and mouse genomic DNA. Figure 6 shows an example of this hybridization. For both bovine and human adrenocortical tissue the parenchymal tissue is formed from cells of the transplanted species, and the blood vessels are of host origin.

Electron microscopic examination of the tissue formed by transplantation of bovine adrenocortical cells showed that the cells have ultrastructural characteristics of the normal adrenal cortex. Two specific features were observed and are shown in Figure 7. One is the presence of microvilli, which are found on the surface of steroidogenic cells that use plasma lipoproteins for the synthesis of steroids from cholesterol. A second feature is the presence of tubulo-vesicular cristae in the mitochondria, characteristic of mitochondria in steroidogenic cells; the mitochondria carry enzymes of part of the pathway of steroid biosynthesis. Ultrastructurally, adrenocortical cells in subcutaneous transplants resembled cells transplanted beneath the kidney capsule (5). Collagen fibers were observed between some cells, but most cells were in direct contact without an extensive matrix between them (Figure 7).

Discussion

The present results show that both bovine and human adrenocortical cells can successfully form functional tissue when transplanted in a subcutaneous site, thereby demonstrating that the subrenal capsule site that we previously used for adrenocortical cell transplantation is not uniquely suited for adrenocortical cell survival and function. In particular, the production of aldosterone by both subcutaneous transplants and subrenal capsule transplants suggests that there is no direct effect of factors at high concentration in the kidney, such as renin, on aldosterone synthesis. Steroid levels produced by adult human adrenocortical cells transplanted in collagen gel were lower but nevertheless sufficient for the healthy survival of adrenalectomized animals. However, for reasons that

are not clear, fetal adrenocortical cells failed to support the life of adrenalectomized mice, and did not form a well-developed tissue structure, despite readily detectable cell division.

Bovine adrenocortical cells, adult human adrenocortical cells and fetal adrenocortical cells all supported angiogenesis, the invasion of the tissue by host endothelial cells. Although all three types of cells were co-transplanted with mitomycin C-treated FGF-secreting 3T3 cells, the patterns of capillary growth differed in the tissues formed. Bovine cells supported a pattern of capillary growth that results in a tissue closely resembling the normal adrenal cortex. In the case of adult human adrenocortical cells, the capillaries that were formed were larger than normal and accumulations of red blood cells were observed in fixed tissue sections. The reasons for the differences between the species are unclear.

We hypothesize that a common factor in subcutaneous and subrenal capsule cell transplantation is the provision of a microenvironment for the transplanted cells that allows survival long enough for vascularization to occur. Studies of the early events in cells transplanted in the kidney show that endothelial cells are found in the transplanted tissue as early as 4 days following transplantation (21), but obviously there is a long period during which the cells must survive without a direct connection with the blood of the host animal. When cells are transplanted subcutaneously as a suspension in culture medium, they probably lack sufficient fluid to bathe the cells with nutrients and oxygen required for their survival. In contrast, the extracellular environment within organs such as the kidney is fluid-rich and has been shown to support survival of transplanted tissues and cells (2, 3, 22). Collagen gel appears to a successful vehicle for subcutaneous transplantation because it maintains the cells in a viable state, presumably by acting as a hydrogel (23), and because it is permissive for the ingrowth of blood vessels. In a small number of animals we also tested transplantation of bovine adrenocortical cells in Matrigel, the extracellular matrix derived from a chondrosarcoma (24). In these animals the transplanted cells survived and were functional as evidenced by plasma cortisol. However, vascularization was limited and the cells remained as isolated groups surrounded by matrix (unpublished observations).

Although the use of collagen gel permitted cell survival and formation of adrenocortical tissue, collagen fibers were not a prominent feature of the tissue when

observed by electron microscopy. Based on our observations in both subcutaneous and subrenal capsule transplantation, we hypothesize that a primary factor in the formation of a tissue structure is the initial aggregation of the cells. A common feature of both subrenal capsule cell transplantation and transplantation in collagen gel may be that these microenvironments are permissive for cell aggregation. When adrenocortical cells are permitted to aggregate in a microenvironment that provides adequate extracellular fluid and appropriate nutrients and oxygen, they have the potential for tissue formation. Attachment of the cells to a surface is neither required nor desirable (6).

In summary, collagen gel is a useful vehicle for the successful subcutaneous transplantation of adrenocortical cells, presumably by protecting the cells during the early phase before vascularization occurs. Subcutaneous transplantation of bovine and human adrenocortical cells provides a useful model for the study of the physiology, cell biology, and molecular biology of these cells in a three-dimensional vascularized tissue structure in a host animal.

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Figure 1

Steroid levels in adrenalectomized *scid* mice receiving transplants of bovine and human adrenocortical cells. 62 animals received transplants of bovine adrenocortical cells. Of these, 10 animals received transplants on the day of adrenalectomy and all others on the day following adrenalectomy. 48 animals survived beyond 20 days following cell transplantation. The graph shows the plasma steroid levels in long-term surviving animals sampled at day 20. Of 12 animals that received transplants of adult human adrenocortical cells, 10 survived beyond 20 days. Animals receiving fetal human adrenocortical cells did not usually survive over the long term; 4 of 13 animals survived beyond 20 days. For all animals blood samples were taken in the late afternoon. On the far right cortisol levels are plotted for 5 individual animals bearing bovine adrenocortical cell transplants sampled at two different times of day, indicating that the circadian rhythm of glucocorticoid levels is maintained.

Figure 2

Gross appearance of a nodule of adrenocortical tissue formed by transplantation of bovine adrenocortical cells in collagen gel. (a) Overview of the subcutaneous location of the tissue in the vicinity of a mammary fat pad. (b) Enlargement showing the intense vascularization achieved.

Figure 3

Nodules of adrenocortical tissue formed by transplantation of bovine adrenocortical cells in collagen gel, showing the effect of the inclusion of FGF-secreting 3T3 cells. (a) Normal appearance of a nodule formed by transplantation of a 5:1 mixture of bovine adrenocortical cells with mitomycin C-treated FGF-secreting 3T3 cells, 14 days following transplantation; (b) necrotic appearance of a nodule formed by transplanting bovine adrenocortical cells without 3T3 cells. Magnification x 50.

Figure 4

Histology and histochemistry of a nodule of tissue formed by transplantation of bovine adrenocortical cells in collagen gel. (a) Low-power view showing the characteristic arrangement of bovine adrenocortical cells into cords typical of the zona fasciculata. Magnification x 50. (b) Staining with *Griffonia* lectin showing invasion of the tissue by endothelial cells. Magnification x 400. (c) Dividing cells demonstrated by staining for Ki-

67 antigen. Magnification x 400.

Figure 5

Histology and histochemistry of nodules formed from human adrenocortical cells transplanted in collagen gel. (a) Nodule of cells formed from adult human adrenocortical cells. The accumulations of many red blood cells mark the large sinusoidal capillaries. Magnification x 50. (a') Lack of proliferating cells as evidenced by staining for Ki-67. Magnification x 200. (b) Nodule of cells formed from fetal adrenocortical cells derived from the definitive zone. Magnification x 200. (b') Proliferating cells stained for Ki-67 antigen. Magnification x 200.

Figure 6

In situ hybridization demonstrating the chimeric nature of tissue formed by transplantation of bovine adrenocortical cells. (a) Hybridization with a bovine satellite I probe shows the bovine origin of the adrenocortical cells. (b) Hybridization with a mouse satellite alpha probe shows the mouse origin of the invading endothelial cells. Serial sections, magnification x 200.

Figure 7

Ultrastructural features of tissue formed by transplantation of bovine adrenocortical cells. (a) Two adjacent cells show interleaved microvilli characteristic of steroidogenic cells. (b) Another cell is adjacent to collagen fibers separated from its neighbor showing the characteristic structure of mitochondria in steroidogenic cells with vesicular cristae. Magnification x 12000.

Formation of normal functional tissue from transplanted adrenocortical cells expressing telomerase reverse transcriptase (TERT)

Michael Thomas, Lianqing Yang and Peter J. Hornsby
Huffington Center on Aging and Department of Cell Biology
Baylor College of Medicine
1 Baylor Plaza M320
Houston, TX 77030

phone 713-798-7334
fax 713-798-6688
e-mail phornsby@bcm.tmc.edu

Abstract

The demonstration that expression of the reverse transcriptase component of human telomerase (hTERT) could immortalize normal cells suggested that such cells could be used for transplantation in gene therapy and tissue engineering. Here we report the first use of hTERT expression in experimental xenotransplantation. Previously we showed that bovine adrenocortical cells can be transplanted into *scid* mice, and that these cells form functional tissue that replaces the animals' own adrenal glands. We co-transfected primary bovine adrenocortical cells with three plasmids, which encode hTERT, SV40 T antigen, *neo*, and green fluorescent protein. Clones were selected by G418 resistance and green fluorescence. These clones do not undergo loss of telomeric DNA and appear to be immortalized. Two clones were transplanted beneath the kidney capsule of *scid* mice. All animals that received cell transplants survived indefinitely despite adrenalectomy. The mouse glucocorticoid, corticosterone, was replaced by the bovine glucocorticoid, cortisol, in the plasma of these animals. The tissue formed from the transplanted cells resembled that formed by transplantation of non-genetically modified cells and was similar to normal bovine adrenal cortex. The tissues are chimeras of bovine cells together with mouse endothelial cells, which form a well-developed vasculature. The proliferation rate in tissues formed from these clones was low and there were no indications of malignant transformation. These experiments show that immortalization with the aid of hTERT enables expansion of cells in culture and that such cells may be transplanted to form a functional tissue that exhibits normal growth control.

Introduction

Most normal somatic cells from humans and other mammals cannot divide indefinitely because of the progressive loss of DNA from telomeres, the specialized ends of the linear chromosomes (1, 2). The gradual erosion of telomeres during repeated cell division results from the combined effects of the inability of normal DNA replication to completely extend telomeric DNA (3, 4) and the lack of expression of the reverse transcriptase component of the telomerase ribonucleoprotein complex (TERT) (5, 6). Stem cells and germ line cells, which normally express TERT and have telomerase activity, are able to fully replicate telomeric DNA and thereby are able to divide indefinitely (7). While cells that do not have telomerase activity are able to divide only a limited number of times before they senesce, in cells with forced expression of TERT the progressive shortening of telomeres is prevented and cells continue to proliferate rapidly at a cumulative population doubling level (PDL) greatly exceeding the PDL at which they would otherwise senesce (8 - 10). These results indicate that expression of TERT is sufficient to immortalize at least some cell types that normally undergo cellular senescence. Moreover, immortalization resulting from TERT expression does not appear to be accompanied by the acquisition of characteristics of cancer cells, such as chromosomal abnormalities, anchorage-independent growth in culture, loss of normal cell cycle checkpoints, or tumorigenicity in immunodeficient mice (11 - 13). Such findings encouraged speculation that cells expressing TERT could be used in cell-based therapies, such as *ex vivo* gene therapy, tissue engineering, and transplantation of cells to correct metabolic or endocrine defects (8, 12, 14, 15). It was recognized that the fulfilment of this promise would depend on ectopic expression of TERT not affecting cellular regulatory systems such as those that control differentiation (14).

In the present experiments we used an adrenocortical cell transplantation model to address this question. Like human cells, bovine cells go through phases of rapid growth, slower growth and eventual senescence in culture, and, unlike rodent cells, very rarely undergo spontaneous immortalization (16). We previously showed that finite-life span clones of bovine adrenocortical cells can be transplanted into *scid* mice, and that these cells form functional tissue that replaces the animals' own adrenal glands, which are removed in the surgical procedure (17). Here we used this cell transplantation model to show that TERT-modified bovine adrenocortical cell clones behave like their normal counterparts, and form functional tissue after transplantation that is histologically similar to tissue formed from normal cells and

shows a similar rate of cell division.

Results and Discussion

Primary bovine adrenocortical cells were co-transfected with three plasmids: pCI-neo-hTERT, in which hTERT is under the control of the CMV promoter (9); pSV3neo, which encodes SV40 T antigen under the control of its own promoter (18); and pEGFP-N1, which encodes enhanced green fluorescent protein under the control of the CMV promoter. All three plasmids also encode *neo*, under the control of the SV40 promoter. We previously showed that transfection with pSV3neo causes an increase in proliferative potential of bovine adrenocortical cells (19); our reason for including it in the present experiments is that we found that when any plasmid is transfected into bovine adrenocortical cells, without pSV3neo as a co-transfected plasmid, cells take up and express the transfected gene but these cells do not grow (unpublished data). Therefore, in the present experiments, we have compared normal (non-genetically modified) bovine adrenocortical cell clones to clones transfected with the combination of pCI-neo-hTERT, pSV3neo and pEGFP-N1, or with the combination of pSV3neo and pEGFP-N1.

Transfected clones were selected for resistance to the antibiotic G418 and for green fluorescence when observed by fluorescence microscopy. Almost all clones showed a positive reaction in the TRAP (telomerase repeat activity protocol) assay. Two clones transfected with hTERT (#47 and #56) were investigated in detail. The TRAP reaction on cell extracts from these clones is shown in Figure 1. Clones of cells grown without genetic modification (e.g. clone #14, which was previously used for cell transplantation (17)) and clones derived with pSV3neo and pEGFP-N1 only were negative in the TRAP assay.

Clone #47 has been grown continuously in culture since its derivation and appears to be immortalized; at the present time it has divided >200 times without senescence and without changes in growth rate. Although the relationship between telomere length and replicative senescence has not been investigated in bovine cells, the similarity of their culture life history to that of human cells suggests that their finite replicative capacity results from telomere shortening. Non-genetically modified bovine adrenocortical cell clones may achieve maximum PDLs of up to ~80 (20, 21). We investigated the effects of hTERT expression on telomere status by hybridizing Southern blots of *Hin*fI-digested DNA with a telomere repeat probe. In DNA from bovine adrenocortical cells freshly isolated from adrenal glands and in

bovine white blood cells the probe hybridized to telomeric restriction fragments (TRFs) of 10 to 30 kb, as previously reported for bovine tissues (22) (Figure 2). DNA from non-genetically modified clones showed a much less intense hybridization in the range of >10 kb. The probe hybridized to discrete smaller fragments in a clone-specific manner. These data are consistent with the hypothesis that erosion of telomeric DNA in normal clonal bovine adrenocortical cells results in their finite replicative capacity, as previously established for human cells (3, 4). An hTERT-modified clone (#47) retained a strong hybridization of TRFs in the range of 10 to 30 kb when assayed at PDLs 53, 83, and 130, as well as showing a clone-specific pattern of smaller fragments (Figure 2).

We tested the ability of clones #47 and #56 to form functional tissue when transplanted into *scid* mice. The clones were expanded in culture and were first used when they had reached a population size suitable for transplantation (PDL 30). Cells were injected into a small polycarbonate cylinder inserted beneath the kidney capsule. During the cell transplantation procedure the animals' own adrenal glands were removed. Following surgery, mice with transplanted cells were observed for 36 days, when they were sacrificed to allow histological studies of the tissue formed from the cells. Adrenalectomy usually results in death within 7 days because of the loss of essential adrenocortical steroids (17). All animals that received cell transplants of clone #47 (PDL 30) and clone #56 (PDL 30) survived in good health to 36 days. The mouse glucocorticoid, corticosterone, was replaced by the bovine glucocorticoid, cortisol, in the plasma of these animals. Data on cortisol levels in mice with transplanted cells are shown in Figure 3. Cortisol is diagnostic of function of the transplanted cells because mice do not make cortisol; the mouse adrenal cortex synthesizes corticosterone as the major glucocorticoid rather than cortisol (17). Cortisol secretion is also the most sensitive measure of cell function because it depends on the formation of a vascularized tissue structure and can only be synthesized if the cells express a complete set of steroidogenic enzymes. Levels of cortisol in mice with transplants of clone #47 (PDL 30) were similar to those in mice with transplants of non-genetically modified clone #14 (PDL 25) (Figure 3). Among 4 clones transfected with pCI-neo-hTERT, pSV3neo, and pEGFP-N1 two (#47 and #56) were transplantable, i.e. able to form functional tissue that produced cortisol and maintained the life of adrenalectomized animals. The other two were not transplantable, i.e., mice with transplanted cells of these clones died before 14 days and at the site of cell transplantation we observed only dead cells or connective tissue. Previously, we showed that 5 of 20 non-

genetically modified clones were transplantable (17). Some clones (4/12) that were derived by transfection with pSV3neo and pEGFP-N1 were also transplantable. Figure 3 shows data for a pSV3neo-modified clone that gave higher cortisol levels than other clones of this type. The figure also shows data for mice with transplants of these different type of cells at higher PDLs. For clone #47, cells were transplantable at PDL 68 and PDL 134, although at these PDLs cortisol levels were lower and some animals died before 14 days. However, these cells are able to form functional tissue at higher PDLs, whereas this was not true for non-genetically modified clones or for pSV3neo-modified clones (Figure 3).

The tissue formed from the transplanted hTERT-modified cells resembled the tissue formed by transplantation of non-genetically modified cells and had a similar histological appearance to normal bovine adrenal cortex. Examples of the tissues formed are shown in Figure 4. The tissues are chimeras of bovine cells together with mouse endothelial cells, which form a well-developed vasculature. Blood vessels supplying the tissue were observed to enter via the kidney capsule, as previously observed in transplants of non-genetically modified cells (17). In the normal bovine adrenal cortex cells are arranged into cords lined by large capillaries. In the tissue formed from both normal adrenocortical cell clones and from hTERT-modified cells, cord-like structures were less obvious, but, as in the normal cortex, each cell was in contact with endothelial cells on one or more surfaces. In the lower part of the transplant tissue adrenocortical cells were in close contact with the kidney parenchyma. There was no evidence of an immune reaction, consistent with the absence of T- and B-cell mediated immunity in the *scid* mouse (23).

The cell proliferation rate in tissues formed from hTERT-modified cells was low. Before transplantation, hTERT-modified clones in culture had a population doubling time of 20-24 hours, similar to that in the exponential growth phase in primary cultures of normal bovine adrenocortical cells (16). This indicates that most cells in hTERT-modified clones are continuously in the cell cycle. Finite life span cells such as clone #14 have a greater doubling time in cell culture (~30 hours) and show a progressive increase in doubling time as the cells approach senescence (20, 21). However, both non-genetically modified cells and hTERT-modified clones showed a much lower proliferation rate in the tissue formed from the transplanted cells (Figure 5). Ki-67 labeling indices were measured in tissue samples from transplanted clonal cells and in samples of normal bovine adrenal cortex. In the adrenal cortex 7.8% of cells were Ki-67$^+$; 2.3% in tissues from clone #47; 5.4% in tissues from clone #56; and

2.4% in tissues from clone #14. When bovine adrenocortical cells proliferating in culture are embedded and sectioned 40 to 50% of cells express Ki-67 when assessed by the same protocol (17). The potential expression of SV40 large T antigen resulting from transfection with pSV3neo was also investigated by immunohistochemistry. However, less than 1% of nuclei in the transplant tissue were immunoreactive for T antigen, whereas >90% of nuclei were immunoreactive in a positive control tissue (liver from a mouse transgenic for SV40 T antigen under the control of the α-1-antitrypsin promoter, kindly provided by M. Finegold) (24). In tissues formed from both hTERT-modified cells and normal clones there were no indications of malignant transformation, such as cells with atypical nuclei or focal areas of higher proliferation rate. The size of the tissue formed from the transplanted hTERT-modified cells at 36 days was not greater than the size of tissue formed from normal clones. In contrast, when cells such as MCF-7 breast cancer, which have an established tumorigenic potential, were transplanted in subrenal capsule cylinders, by 36 days the tumor tissue had expanded outside the cylinder and ~35% of the cells were Ki-67$^+$.

Our data show that the expression of hTERT, producing active telomerase, can be used to expand endocrine cells in culture for subsequent transplantation. This will be valuable in basic studies of cell proliferation and differentiation as well as in potential therapeutic procedures. The data obtained on the tissues formed by cell transplantation are consistent with the maintenance of normal function and growth control in such cells. The ability to synthesize cortisol and to support the life of adrenalectomized animals indicates that the patterns of differentiated gene expression in cell transplant tissues must be similar to that of the normal adrenal cortex, although an extensive investigation will be required to validate this conclusion in detail. TERT expression allows indefinite growth of cells and may reduce the occurrence of changes during cell culture expansion that alter the potential of the cells to resume normal function and growth control after reintroduction into the *in vivo* environment.

Methods

Derivation of hTERT-modified clones. Plasmids used for transfection were pCI-neo-hTERT (9), generously supplied by R. Weinberg, pSV3neo (18), and pEGFP-N1, a plasmid encoding enhanced green fluorescent protein (Clontech). Primary bovine adrenocortical cells were prepared by enzymatic digestion of adult adrenal tissue (25). Cells growing in primary culture were co-transfected with the three linearized plasmids at a ratio of 1 μg: 1μg: 0.4 μg using

Effectene (Qiagen) following the manufacturer's instructions. Clones that were selected for further study were resistant to 300 μg/ml G418 and showed uniform green fluorescence. Clones were expanded to ~10^7 cells for transplantation and biochemical studies.

Assay for telomerase activity. Cells were harvested using bacterial protease (Type XIV, Sigma) (25) and were extracted in deoxycholate/NP-40 buffer (26). The TRAP (telomerase repeat activity protocol) assay (27) was performed using the TRAP-eze kit (Intergen Co., Gaithersburg, MD). Each cell extract was diluted to 1, 0.5, and 0.25 μg protein per reaction and was incubated with ^{32}P-end labeled TS primer in TRAP buffer for 10 minutes at 30 °C. The tubes were then incubated at 80 °C, and a mixture of extended CX primer, internal control template and internal control primer were added, plus Taq polymerase. PCR was performed for 31 cycles. TRAP reaction products were separated by non-denaturing electrophoresis on 10% polyacrylamide.

Southern blotting of telomeric restriction fragments. Genomic DNA extracted from cells and tissues (28) was digested with *Hin*fI (10 U/μg, New England Biolabs) for 16 hours at 37 °C and the digestion products were separated by electrophoresis in 1% agarose (29). Following blotting onto uncharged nylon membranes (Pall Biodyne) and UV-crosslinking, DNA fragments were hybridized with random-primer labeled insert from plasmid pHuR93, which comprises 40 tandem TTAGGG repeats (30). Membranes were washed in 0.1 x SSC at 65 °C and exposed to X-ray film. Even loading of the lanes was assessed by ethidium bromide staining of the agarose gel and by re-probing the membranes with bovine satellite I (29).

Cell transplantation. 2 x 10^6 cells per animal were transplanted beneath the kidney capsule of *scid* mice together with 0.4 x 10^6 irradiated FGF-secreting 3T3 cells (17). Under tribromoethanol anesthesia, mice were adrenalectomized and cells were transplanted in a single procedure. A small polycarbonate cylinder was used to create a virtual space beneath the kidney capsule into which the cells could be introduced. A 1-mm length of polycarbonate tube (3-mm internal diameter) was surface-polished by brief exposure to dichloromethane vapor. The cells were introduced into the subcapsular cylinder by a transrenal injection using a 50 μl Hamilton syringe with a blunt 22 gauge needle. The cells are then confined to a space bounded laterally by the walls of the cylinder and on the top and the bottom by the capsule and parenchyma of the kidney respectively. Post-operative care for the animals consisted of the administration of synthetic steroids and the administration of analgesics and antibiotics in the drinking water. For seven days after surgery, animals were injected subcutaneously once daily

with 1 μg/g body weight fludrocortisone acetate and dexamethasone phosphate. One week after cessation of the steroid administration, and at weekly intervals thereafter, tail blood samples were taken 15 minutes after the injection of ACTH (Sigma, 0.01 units per gram body weight). Animals were sacrificed 36 days after cell transplantation.

Histology and immunohistochemistry. For histological examination the cylinder used for cell transplantation was removed and the tissue was fixed in paraformaldehyde, dehydrated, and embedded in paraffin (17). Sections (5 μm) were stained with hematoxylin and eosin. Bovine and mouse cells were distinguished by fluorescence microscopy on the basis of different patterns of reaction of chromatin in deparaffinized sections with DAPI (4′,6-diamidino-2-phenyl indol, Sigma) (17). The proliferation-associated Ki-67 antigen was detected using monoclonal antibody MIB-1 (Coulter Cytometry, Miami, FL) and a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA). This procedure stains only proliferating cells in the transplant tissue because the antibody does not react with mouse Ki-67.

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Figure 1

Telomerase activity by TRAP assay in bovine adrenocortical cells expressing human telomerase reverse transcriptase (hTERT) and in control clones. Clones #47 and #56 were derived by transfection of primary bovine adrenocortical cells with pCI-neo-hTERT, pSV3neo and pEGFP-N1. Clone #14 is a non-genetically modified clone previously used for cell transplantation (17). Two other clones were derived by transfection with pSV3neo and pEGFP-N1 without pCI-neo-hTERT. For each clone, cell extracts at 1, 0.5 and 0.25 μg protein were assayed. Amplification of the internal control fragment (marked with an asterisk) tests for possible inhibition of the PCR stage of the TRAP assay, which can produce false negatives. Clone #47 was assayed at two population doubling levels (PDL); **a** PDL 55 and **b** PDL 83. Clone #56 was at PDL 25; clone #14 at PDL 30; and the two pSV3neo-only clones were at PDL 25. "0" are reactions with cell lysis buffer only.

Figure 2

Telomere restriction fragment (TRF) analysis in hTERT-modified bovine adrenocortical cells and in control cells. Telomere status was assessed by Southern blotting of *Hin*fI-digested DNA and hybridization with a telomeric repeat probe. DNA was isolated from clone #47 at three different PDLs and from two non-genetically modified clones (#14 and #5; both at PDL 30) and a senescent subclone of clone #5 (clone #5b, PDL 55). DNA was also prepared from bovine white blood cells and from freshly dissociated cells of the adrenal cortex. The numbers on the left show the positions of marker DNA fragments in kb.

Figure 3

Levels of plasma cortisol in mice with transplanted cells. Each set of connected open circles represents data on an animal with transplanted cells of the indicated type. The first symbol of each set shows the cortisol level in plasma from blood sampled at 14 days after cell transplantation. In some animals blood samples were then taken at weekly intervals thereafter. For clones #47, #14, and #21 data are also shown for mice with cells transplanted at higher PDLs. For all three clones at higher PDLs, 25-50% of mice died before the first blood samples were taken and these mice are not represented on the figure. When clones #14 and #21 were transplanted at PDLs >35, all mice died before 14 days. Other non-transfected clones or clones transfected with pSV3neo plus pEGFP-N1 showed similar behavior.

Figure 4

Histological appearance of tissues formed by transplantation of hTERT-modifed bovine adrenocortical cell clones and normal adrenocortical tissue. a, b, examples of tissue formed by transplantation of clone #47; c, d, examples of tissue formed by transplantation of clone #56; e, f, tissue formed from transplanted cells of a non-genetically modified clone, #14; g, h, normal bovine adrenal cortex. In A-F the mouse kidney may be seen below the transplant tissue. Hematoxylin and eosin stain, magnifications x 250 (a, c, e, g) and x 400 (b, d, f, h).

Figure 5

Proliferation in tissues formed by transplantation of hTERT-modified bovine adrenocortical cell clones assessed by expression of Ki-67 antigen. a, tissue formed by transplantation of clone #47; b, tissue formed by transplantation of clone #56; c, tissue formed from transplanted cells of a non-genetically modified clone, #14; d, normal bovine adrenal cortex. Magnification x 300.

Early events in the formation of a tissue structure from dispersed bovine adrenocortical cells following transplantation into *scid* mice

James R. Tunstead, Michael Thomas, and Peter J. Hornsby
Huffington Center on Aging and Department of Cell Biology
Baylor College of Medicine
Houston, TX 77030
USA

For Correspondence:

Peter J. Hornsby, Ph.D.
Huffington Center on Aging
Baylor College of Medicine
1 Baylor Plaza M320
Houston, TX 77030
Tel: 713-798-7334
Fax: 713-798-6688
E-mail: phornsby@bcm.tmc.edu

Running head: Transplantation of bovine adrenocortical cells

Abstract

The early events that follow the transplantation of dispersed bovine adrenocortical cells into *scid* mice were investigated. Adrenocortical cells were introduced into a small cylinder inserted beneath the kidney capsule, where they form a tissue structure that becomes vascularized and secretes steroids that replace those from the animal's own adrenal glands, which are removed during the transplantation surgery. We studied cell proliferation, cell survival, apoptosis, and the role of p21WAF1/CIP1/SDI1 over the first 6 days following transplantation. Additionally we examined the invasion of the tissue by host macrophages and endothelial cells. The data show that there is healthy survival of most of the transplanted cells and that this is related to their position in the cell transplantation cylinder. In the layer of cells that was adjacent to the kidney parenchyma there was a higher rate of cell proliferation and a lower rate of apoptosis than in cells that were located in the upper part of the cylinder. In the lower layer cells were more likely to have nuclear p21, and macrophages and endothelial cells were observed only in this region. Cells that incorporated bromodeoxyuridine administered to animals 2 or 4 days following transplantation were not more likely than other cells to be undergoing a second division when the animals were sacrificed at 6 days, suggesting that proliferation in the lower layer is not confined to a small subpopulation of cells. Among different animals, the extent to which the spaces between the transplanted cells became lined by host endothelial cells correlated with higher levels of proliferation and nuclear p21, suggesting that vascularization is the critical step for the continued survival and proliferation of the transplant. The present experiments show that bovine adrenocortical cells transplanted into *scid* mice form a useful model for the study of tissue formation from dispersed cells and the interaction of the transplanted cells with the host.
Keywords: Adrenocortical cells, proliferation, apoptosis, vascularization

Introduction

Protocols for cell transplantation have been developed both for basic research and for therapeutic purposes, such as the correction of hormonal or metabolic defects and *ex vivo* gene therapy. The events immediately following introduction of the transplanted cells into the host have been rarely studied, even though these are critical for the survival of the cells and for the eventual success of the transplantation procedure. In previous work from this laboratory we showed that primary bovine and human adrenocortical cells, as well as clonal bovine adrenocortical cells, may be transplanted into immunodeficient *scid* mice, where the cells form functional vascularized tissue (1 - 3). The animals' own adrenal glands are removed during transplantation of the adrenocortical cells. Steroids secreted by the transplant rescue the animals from the normally lethal effects of adrenalectomy. These experiments show that it is possible for a xenotransplanted cell type to replace the function of a surgically removed endocrine gland (1 - 3). Although we usually sacrificed the animals after 30-40 days, we have also shown that the transplant survives and functions to keep the host animal alive and healthy for at least 300 days (2). Transplantation of adrenocortical cells has potential applications both in basic science studies and in cell therapy; although a direct application would be the correction of adrenocortical insufficiency, a more generally applicable therapeutic use would be the delivery of proteins by genetically modified adrenocortical cells (4).

During these experiments it became clear that the critical period for the success of the cell transplantation procedure is the first week following introduction of the cells into the host animal. During that time the dispersed cell suspension undergoes a transition to a tissue structure. This process has received only minimal attention in cell transplantation methods generally. The biology of the cells growing in culture and the biology of the cells in a 3-dimensional vascularized functional tissue structure *in vivo* are evidently very different. To understand the process of cell transplantation and to improve the technology it is important to understand how cells interact with each other and cooperate with host elements in the process of forming a tissue structure from dispersed cells.

In the method we used, adrenocortical cells are introduced into a small cylinder inserted beneath the kidney capsule, providing a reproducible technique (1 - 3). The cells

are confined to this chamber and do not disperse into the host tissue, thus simplifying retrieval of the cells at intervals following transplantation for examination of their structure and function. The cells do not require to be attached to a polymer matrix in order to be able to form a tissue structure within the transplantation chamber. Indeed, their attachment to a matrix was detrimental to this process *in vivo* (2). The cells apparently require to be introduced into a space sufficient to allow aggregation and the subsequent formation of a vascularized functional tissue.

The present experiments were designed to provide basic information on events occurring over the first few days following transplantation of bovine adrenocortical cells. Bovine cells were used because their ready availability as primary cell populations makes them ideal for this kind of study; data obtained with bovine cells should be applicable to subsequent basic and clinical studies with human cells. This information forms the basis for elucidating the factors that affect cell survival, cell proliferation, and the cooperation of the transplanted cells with host cells, such as endothelial cells and macrophages. We also studied the expression of the cell cycle inhibitor $p21^{WAF1/CIP1/SDI1}$ (5 - 7). We previously observed that bovine adrenocortical cells in culture, throughout their life span, maintain relatively high levels of p21 (8). Labeling studies, however, show that cells in the S phase of the cell cycle do not express p21; p21 is expressed in the G1 and G2 phases of the cell cycle (9). These observations are consistent with a model in which expression of p21 is involved in controlling the end of one cell cycle and the decision by the cell to enter a new cell cycle or to leave the cell cycle (9). Interestingly, in the adrenal cortex *in vivo* in young animals there is almost no expression of p21 (8, 10), despite a readily detectible rate of cell division (1). Therefore p21 appears to be involved in the regulation of the cell cycle in culture but not *in vivo*. We wished to observe the role of p21 in cells that are making the transition from cell culture back to an *in vivo* tissue structure in these experiments.

Methods

Growth of bovine adrenocortical cells in culture

Bovine adrenocortical cells were derived by enzymatic and mechanical dispersion from the adrenal cortex of two-year-old steers, as previously described (11, 12). Primary cell suspensions were stored frozen in liquid nitrogen.

Frozen cells were thawed and plated in Dulbecco's Eagle's Medium/Ham's F-12 1:1 with 10% fetal bovine serum, 10% horse serum and 0.1 ng/ml recombinant basic FGF (Mallinckrodt, St. Louis, MO) (11, 12). Cells were grown in culture for 7 days before transplantation.

FGF-transfected 3T3 cells

In the experiments previously reported, we transplanted bovine adrenocortical cells together with a line of 3T3 cells stably expressing fibroblast growth factor-1 (FGF-1) fused in frame with a signal peptide from hst/KS3, yielding a highly angiogenic secreted product (13) (generously supplied by T. Maciag). When these cells were omitted from the transplant, the development of the tissue, particularly angiogenesis, was impaired (1, 3). 3T3 cells were grown under the same conditions as bovine adrenocortical cells. To render the 3T3 cells incapable of further division after transplantation, the cells were incubated at ~20% confluence for 24 hours with 2 μg/ml mitomycin C (Sigma, St. Louis, MO) (1, 3).

Transplantation of cells beneath the kidney capsule of scid *mice*

ICR *scid* mice originally purchased from Taconic (Germantown, NY) were maintained in an animal barrier facility as a breeding colony. Animals at an age greater than 6 weeks (~25 g body weight) were used in these experiments. Procedures were approved by the institutional animal care committee and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Under tribromoethanol anesthesia, mice were adrenalectomized and were transplanted with adrenocortical cells in a single procedure. A longitudinal incision was made with fine scissors in the dorsal skin. A 1.5-cm incision in the lateral body wall was made to open the retroperitoneal space. Adrenalectomy was performed on both sides.

In order to confine the transplanted adrenocortical cells within a defined space so that the growth, vascularization and function of the cells could be readily studied, we used a small cylinder to create a virtual space beneath the capsule into which the cells

could be introduced (1, 3). In these experiments, cylinders were formed from the biocompatible copolymer poly(lactide-co-glycolide) (PLGA, M. W. ~100,000, Birmingham Polymers Inc., Birmingham, AL). The polymer was dissolved in chloroform and cylinders were formed by building up thin layers by evaporation in a Teflon mold. As the chloroform evaporated, further layers of polymer were added until a cylinder 1.5 mm in height and 3 mm internal diameter was formed. Paraffin-embedded polymer cylinders may be sectioned using a disposable microtome blade.

Using the incision in the body wall prepared for adrenalectomy, the left kidney was exteriorized and a small transverse incision was made through the capsule on the ventral surface of the kidney near the inferior pole. Using one point of a fine forceps, a pocket was created under the capsule. A cylinder was pushed partially into the pocket under the capsule, filled with culture medium, and then introduced fully into the pocket so that the capsule on the top and the kidney parenchyma on the bottom formed a sealed space.

Adrenocortical cells were introduced into this space as follows. Cells were released from the culture dish by digestion with bacterial protease (11, 12). 2.5×10^6 adrenocortical cells, and 5×10^5 mitomycin C-treated 3T3 cells were injected in each animal.

The cell suspension was pelleted and kept on ice. For injection, the pellet was resuspended in medium, the total volume of the suspension being just greater than the volume of the pelletted cells. The cells were introduced into the subcapsular cylinder by a transrenal injection using a 50 μl Hamilton syringe with a blunt 22 gauge needle. Following transplantation of the cells, the kidney was returned to the retroperitoneal space, bathed in ~3 ml PBS, the body wall was closed with 6-0 nylon sutures and the skin closed with surgical staples. Animals were maintained at 35 °C ambient temperature until recovery from the anesthetic.

Post-operative care for the animals was the same as that previously used in experiments in which animals were permitted to survive over longer periods than in the present studies. Animals were injected subcutaneously once daily with 1 μg/g body weight fludrocortisone acetate and dexamethasone phosphate (1) and were given access to drinking water containing 1 mg/ml acetaminophen, 0.1 mg/ml codeine, 1 mg/ml

tetracycline, 1 mg/ml sulfamethoxazole, and 0.1 mg/ml trimethoprim. Although steroid treatment was used because this prevents some early deaths resulting from the loss of steroids by adrenalectomy (1), we subsequently found that this treatment may be omitted without affecting the eventual outcome (3). Therefore it is unlikely that the steroids affect events such as cell proliferation that are important in the formation of the transplant tissue.

Animals were sacrificed 1, 2, and 6 days following cell transplantation (3, 3, and 11 animals respectively). Animals to be sacrificed at 2 and 6 days were given a single intraperitoneal injection of bromodeoxyuridine (BrdU, Sigma) at 50 mg/kg body weight 24, 48, or 96 hours before sacrifice.

Histology and immunohistochemistry

The tissues formed from transplanted cells were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (6 μm) were deparaffinized and rehydrated using graded alcohol concentrations. Antigen retrieval was performed by heating at 100 °C in 10 mM sodium citrate pH 6.0 for 15 min. After blocking nonspecific binding with horse serum for 30 min., sections were incubated with primary antibodies for 16 hours at 4 °C as follows: rat monoclonal anti-BrdU, clone BU1-75 (Harlan, Indianapolis, IN), at 1:75 dilution; mouse monoclonal MIB-1 against Ki-67 antigen (Coulter Cytometry, Miami, FL) at 1:100 dilution; mouse monoclonal anti-human p21, clone EA10 (Oncogene Research Products, Cambridge, MA) (14), which recognizes bovine p21 (10), at 1:100 dilution; rat monoclonal anti-mouse platelet endothelial cell adhesion molecule (PECAM1), clone MEC 13.3 (Pharmingen, San Diego, CA) at 1:100 dilution; mouse monoclonal anti-F4/80 mouse macrophage marker, clone Cl:A3.1 (Harlan, Indianapolis, IN) at 1:75 dilution. Unbound antibody was removed by washing in PBS at room temperature and then a horse anti-mouse polyclonal antibody conjugated with biotin (Vector Laboratories, Burlingame, CA) was added at 1:75 dilution for 1 hour at room temperature. For fluorescence microscopy, the bound antibody was visualized by addition of avidin-fluorescein conjugate or avidin-Texas Red conjugate (Vector Laboratories) at 4 μg/ml in 50 mM sodium bicarbonate, 15 mM sodium chloride, pH 8.2, for 45 minutes. Sections were washed in the same buffer. Alternatively, for light microscopy, the antibody was visualized by an avidin-biotin-peroxidase complex (Vector

Laboratories) as recommended by the manufacturer, and sections were lightly counterstained with hematoxylin.

We used the following protocol to perform double labeling for BrdU and Ki-67. After the sections had been labeled with anti-BrdU, visualized with avidin-Texas Red conjugate, sections were incubated in a biotin blocking solution as recommended by the manufacturer (Vector Laboratories). Incubation of the sections with MIB1 antibody followed by visualization of the antibody with an avidin-fluorescein conjugate was then performed as described above. To perform double labeling for p21 and Ki-67, sections were labeled with anti-p21, visualized with avidin-Texas Red conjugate, and then with a 1:10 dilution of MIB1 antibody which had been directly conjugated to fluorescein (Coulter Cytometry, Miami, FL).

Before observation by fluorescence microscopy, sections were counterstained with a DNA-binding dye. Sections were incubated for 5 minutes at 4 °C in 1 μg/ml 4′,6-diamidino-2-phenyl indole (DAPI). After washing, sections were observed and photographed using a triple band (red/green/blue) emission filter in conjunction with single, double or triple (yellow, blue, and ultraviolet) excitation bands. Numbers of nuclei labeled by the different antibodies used were counted from photographs of random fields; 1000 to 2000 cells were scored for each animal.

Labeling of double strand breaks by ligation of biotin-labeled hairpin oligonucleotides

A hairpin oligonucleotide that can be ligated to double-strand breaks, as found in apoptotic cells, was described previously (15). It has a 10-bp stem region to form a hairpin with a defined double-strand end with a single 3′ A overhang (15). A loop of 20 nt was designed to accommodate biotin labels without base-pairing in this region. At 5 places in the loop the oligonucleotide was synthesized with amino modifier C6 deoxyuridine (Glen Research, Sterling, VA). After synthesis of the oligonucleotide, biotin was covalently attached to the amino groups by reaction with biotin bis-aminohexanoyl N-hydroxysuccinimide ester (Glen Research). The synthesis and post-synthesis biotinylation were performed by Synthetic Genetics Corp. (San Diego, CA).

The oligonucleotide was ligated to DNA in tissue sections *in situ* using T4 DNA ligase. Sections were deparaffinized with xylene and rehydrated in graded alcohol concentrations. After washing in water, sections were incubated for 90 minutes at 65 °C

in 10 mM sodium citrate, pH 6.0, and then re-washed with water.

The following procedures were performed at room temperature (23 °C). Sections were incubated with 25 μg/ml proteinase K (Oncor, Gaithersburg, MD) in PBS for 5 minutes. Sections were then rinsed thoroughly with water. A mix of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 15% polyethylene glycol (8000 m.w., Sigma), with hairpin oligonucleotide at 35 μg/ml and DNA T4 ligase (Boehringer Mannheim, Indianapolis, IN) at 250 units/ml was added (20 μl per section). Sections were covered with glass coverslips and placed in a humidified box for 16 hours. The sections were then washed with several changes of water over 2 hours. The bound biotin on the section was then visualized with an avidin-fluorochrome conjugate as described above.

Dual staining with hairpin oligonucleotide probe and monoclonal antibodies was achieved by first ligating the oligonucleotide probe to the section and visualizing bound biotin with an avidin-Texas Red conjugate. Sections were incubated in a biotin blocking solution as recommended by the manufacturer (Vector Laboratories). Incubation of the sections with the antibody followed by visualization of the antibody with an avidin-fluorescein conjugate was then performed as described above.

Results

We investigated the process by which transplanted adrenocortical cells undergo the transition from a cell suspension, the form in which they are introduced into the host animal, to a true tissue structure. As described in Methods, we transplanted 2.5×10^6 bovine adrenocortical cells and 5×10^5 FGF-secreting 3T3 cells, the latter rendered incapable of division by mitomycin C treatment. The suspension of cells was introduced by a transrenal injection into a small PLGA cylinder inserted beneath the kidney capsule of *scid* mice. Animals were sacrificed at 1, 2 or 6 days after transplantation and the histology of the transplanted cells was examined.

The cells separate into three layers within the cylinder. This was noted at 24 hours following cell transplantation but was more marked at day 6 (Figure 1). A basal layer comprised of aggregated, apparently healthy cells was attached to the renal parenchyma. A layer of smaller aggregates and single cells was present within the center of the cylinder. These cells showed diminished cytoplasmic volume and pyknotic nuclei.

Fig. 1

An upper thin layer of cells of healthy appearance was attached to the the kidney capsule. The upper and middle layers varied with respect to depth and extent of cell aggregation, whereas the basal layer was more solid and was about 10 - 15 cells in thickness.

Over the first few days the transplanted bovine adrenocortical cells were invaded by host (mouse) cells. At 24 hours almost all cells are bovine rather than mouse, based on nuclear staining by DAPI. Mouse cells may be distinguished from those of other species by the presence of several brightly staining points in the nuclei (16). Endothelial cells were detected by immunoreactivity for PECAM1 (17). No PECAM1 staining was seen at days 1 and 2. By day 6 endothelial cells were observed lining spaces between the adrenocortical cells in the basal layer; no endothelial cells were observed in the other layers. The spaces were also observed in the basal layer at earlier times, but endothelial cells were not present (Figure 2). Endothelial cells were observed to be of mouse origin on the basis of therir nuclear staining with DAPI. Additionally, at day 6, but not at days 1 and 2 after transplantation, many macrophages were present in the basal layer. These cells were detected by the F4/80 macrophage-specific marker (18) (Figure 2).

Fig. 2

At day 6, in 7 of 11 transplants examined, endothelial cell-lined spaces were found throughout or in parts of the basal layer of cells. The other 4 transplant tissues showed PECAM1-positive vessels only along the junction between the renal parenchyma and the basal layer of adrenocortical cells. Angiogenesis appeared to be proceeding more rapidly in the former group, and therefore in some of the experiments reported here we divide the animals into two groups based on the extent of endothelial cell invasion. Although we divided the transplants into two groups for the purpose of analysis we have no indication that the ultimate extent of vascularization in these groups would differ, because we have observed that primary bovine adrenocortical cell transplants are uniformly well vascularized when animals are sacrificed at 30-40 days (1, 3). However, the variation in the rate of angiogenesis in the present experiments allowed us to observe factors that are associated with the vascularization process.

We used several histological techniques to examine patterns of cell death and cell proliferation, both with respect to the time following transplantation and with respect to the location of the cells within the transplant. In particular, we used a recently developed specific and sensitive method for the detection of apoptosis, in which a biotin-labeled

hairpin oligonucleotide is ligated to double-strand breaks in nuclei (15). The application of this technique to sections of transplant tissue confirmed that cells within the middle layer, which showed histological features of dying cells, were indeed undergoing apoptosis (Figure 3). On the other hand, the layers next to the kidney and next to the kidney capsule had very few apoptotic cells. At 24 hours the dying cells are bovine, based on the fact that very few mouse cells are present at this time, but at later times the dying cells are of both bovine and mouse origin.

Fig. 3

We also performed several forms of double labeling on the transplant sections. These studies used combinations of oligonucleotide ligation, to detect apoptotic cells, together with immunohistochemistry; the oligonucleotide labeling method permits subsequent immunohistochemistry on the same section (15, 19). Additionally we used immunohistochemistry with two different antibodies (combinations of anti-BrdU, anti-Ki-67, and anti-p21). Ki-67 is an antigen expressed by proliferating cells (20); the anti-Ki-67 antibody MIB-1 does not recognize the mouse homologue of Ki-67 but recognizes the bovine protein with staining equivalent to that observed with human tissues (our unpublished observations). Figure 4 illustrates these double-labeling techniques. Apoptotic cell labeling by oligonucleotide ligation is compatible with subsequent labeling by antibodies, as shown by the fact that the discrete patterns of labeling by the individual techniques were maintained when double labeling was used. Additionally, separate patterns of labeling by antibodies were maintained in the combinations used (anti-BrdU/anti-Ki-67, and anti-p21/anti-Ki-67).

Fig. 4

These double-labeling techniques were used to test hypotheses about the patterns of cell death and cell proliferation within the transplant tissue. First, because of the multiple roles of the cell cycle inhibitor p21, we wished to establish whether p21 labeling was associated with the damaged and dying population of cells within the tissue, or alternatively was associated with the healthy dividing population. Second, we wanted to distinguish between the alternate possibilities that most proliferation within the transplanted cells was occurring in a small subpopulation, or that all the healthy nondying cells were capable of division *in vivo*.

Application of double labeling techniques to the transplant tissues confirmed that many of the cells in the middle and upper layers of the transplant were undergoing

apoptosis (Figures 3a, 4a, and 5a,c). Additionally, few of the cells in these layers were Ki-67$^+$ (Figure 5a) and few p21$^+$ cells were observed in these layers (Figures 4a and 5c). On the other hand, the basal layer showed few cells undergoing apoptosis and most of the Ki-67 and p21-labeled cells were in this layer (Figure 4b,c and Figure 5b,d). These data are consistent with the impression from conventionally stained sections of the tissues that the basal layer contained healthy dividing cells and that the middle and upper layers were dying. Moreover, p21 was clearly not associated with apoptosis but was mostly confined to the healthy layer, although an occasional cell was found to be double labeled by oligonucleotide probe ligation and by anti-p21, as shown in Figure 4a.

Fig. 5

Because the upper layers were composed of dying cells, we concluded that cells in these layers did not contribute to the transplant tissue seen at 30-40 days (1, 3). The following studies were confined to the basal layer which gives rise to the mature transplant tissue structure. In this layer, we wished to examine whether most proliferation was occurring in a small subpopulation, or whether all the healthy nondying cells can divide *in vivo*. To study this, BrdU was administered to some animals 1 to 4 days before sacrifice (Figure 6). Between 4% and 8% of cells were labeled when the animals were sacrificed 1 to 4 days later. Because a cell labeled in S phase would be expected to produce two labeled nuclei after cell division, this gives upper values of 2% to 4% of cells in S phase at the time of BrdU administration. It is interesting to note that the value obtained by labeling 4 days before sacrifice and the value obtained by labeling 2 days before sacrifice were very similar. If a cell divides continuously with a 24-hour cell cycle, it could give rise to 16 labeled cells 4 days after incorporating BrdU. A cell cycle time of ~24 hours is consistent with data on bovine adrenocortical cells in primary culture (9). When double labeling with BrdU and Ki-67 was performed, those cells which incorporated BrdU 2 to 4 days previously were neither more nor less likely to be in division at the time of sacrifice, as indicated by Ki-67 labeling, than those that did not incorporate BrdU (Figure 6B). However, a few cells do undergo repeated replication, as demonstrated by double staining of some individual nuclei (Figure 4b). When labeling with BrdU was only 24 hours before sacrifice, more BrdU-labeled cells were Ki-67 positive, presumably some cells were still expressing Ki-67 in the same cell cycle as the S phase in which they incorporated BrdU; Ki-67 is expressed over the entire cell cycle in

Fig. 6

dividing cells (20).

We investigated proliferation and p21 expression in the basal layer of the transplant tissue in more detail. Tissues that showed more advanced angiogenesis, determined as described above by PECAM1 staining, were compared with those in which angiogenesis was occurring more slowly. p21 was much higher in the basal layer than in the other layers. Within this layer, levels of nuclear p21 staining in individual cells formed a continuous variable, unlike labeling for Ki-67 or BrdU, making it difficult to divide the population into $p21^+$ and $p21^-$ (Figure 7). When assessed by either of two different detection techniques, p21 levels fell from their highest value at the early phase of the transplant to lower levels at day 6; the fall was greater in those tissues in which angiogenesis was occurring more slowly.

Fig. 7

Ki-67 labeling was also highest in tissues with more extensive angiogenesis (Figure 8A). Double labeling for Ki-67 and p21 showed that both at early and later times within the experiment, and in tissues with different extents of angiogenesis, dividing cells were much more likely than nondividing cells to have a high level of nuclear p21 (Figure 8B). Fortuitously, a cell in metaphase was observed by this double-labeling technique (Figure 9). It is of interest to note that the chromosomes are stained by the antibody against Ki-67, which is a chromatin protein (21). However, p21 staining, which is typically confined to nuclei, was found throughout the cell, consistent the fact that it is not normally bound to chromatin (22).

Fig. 8

Fig. 9

Discussion

In our adrenocortical cell transplantation procedure, a dispersed cell population is introduced into the host animal in a confined space beneath the kidney capsule. The data presented here show that the cells aggregate after transplantation, and that those cells that lie in a basal layer next to the kidney are viable, whereas those further away are not. The upper layers of cells have a high rate of apoptosis and are unlikely to contribute to the mature tissue structure that is observed at 30 days and later (1 - 3). Because we have observed that adrenocortical cells can be transplanted subcutaneously in collagen gel (2, 23) it is unlikely that the kidney has a unique role in maintaining adrenocortical cell viability. We hypothesize that the supply and exchange of extracellular fluid is adequate

nearer the kidney but inadequate at greater distances.

In this environment, adrenocortical cells are able to aggregate to form a mass that later becomes a true tissue structure. During aggregation in the basal layer, spaces form between the cells, which become lined with endothelial cells by 6 days, showing that angiogenesis begins rapidly in the transplant tissue. The initial aggregation of the cells may indicate a form of self-organizing ability. Endothelial cells follow the patterns laid down by this initial aggregation. Over the time studied here there was also an influx of macrophages into the basal layer. The significance of this observation is yet to be determined, but macrophages have been found to have a positive influence on wound healing in various model systems (24).

Cell division occurs only in the basal viable layer. Double BrdU/Ki-67 labeling data show that in this layer proliferation is not confined to a minor cell subpopulation. In this respect, the transplant tissue resembles the population of cells in primary culture, where almost all cells are capable of cell division. As we have discussed elsewhere, there is no evidence for a specific stem cell population in the adrenal cortex (1, 25). The rate is low compared with the rate of proliferation that the cells had in culture before transplantation (9), but it was higher in those tissues that show more extensive invasion by endothelial cells. These data indicate that adrenocortical cells rapidly support angiogenesis and undergo cell division if they are able to survive the period immediately following transplantation. The viable cells are able to cooperate with host cells in the process of angiogenesis, which likely involves more than just the supply of angiogenic factors (26). The newly formed blood supply supports limited cell division, consistent with a growing body of evidence that tissue size and growth *in vivo* are regulated by angiogenesis and not by a direct control of the cell cycle (27). Proliferation of cells in bovine adrenocortical cell transplants continues at a low rate over long periods, although by 300 days it is almost zero (2). The tight growth control in the cells *in vivo* contrasts with their prior status in cell culture, where they divide continuously, driven by the high mitogen content and the stimulatory effect of attachment to tissue-culture plastic. This rapid growth is reversible after cell transplantation, even when the cells are clonal (1), indicating that continuous rapid growth in culture, in the absence of genetic changes, does not prevent the reimposition of normal growth control by angiogenesis when the

cells are placed in an appropriate environment *in vivo*.

Although the cell cycle inhibitor $p21^{WAF1/CIP1/SDI1}$ can be induced by DNA damage in a p53-dependent manner (28), the present experiments show that p21 is mainly associated with cell division rather than cell damage. Expression was higher in the layer of viable cells. In cultured cells, not exposed to DNA damage, p21 is elevated both in G1 and in G2 but declines to very low levels during S phase (29, 30). The relationship between p21 cell division in proliferating bovine adrenocortical cells in culture is consistent with these changes in the cell cycle (9). This suggests that p21 plays a role in the cell cycle in this cell type, perhaps involving the decision to re-enter the cell cycle after following another cycle. However, this possibility must be contrasted with the almost complete absence of p21 expression in the normal bovine adrenal cortex (8, 10), despite a readily detectable rate of cell proliferation in young animals (1). Thus growth control in the transplant in this respect appears to retain some aspects of growth control as it was in cell culture and does not fully resemble the normal gland *in vivo*. However, at 300 days following cell transplantation p21 is almost completely absent, as is cell division (2 and M. Thomas and P.J. Hornsby, unpublished observations). It should also be noted that the very high p21 levels immediately following cell transplantation could reflect temporary DNA damage occurring during the preparation of the cell suspension and the introduction of the cells into the animal. We have previously shown an association between DNA damage, induction of p21 and apoptosis in the rat adrenal cortex *in vivo* (10, 31).

In conclusion, these studies form the basis for future studies of a critical question in cell transplantation, how cells undergo the transition from a dispersed cell population into an intact tissue. Such studies using cell transplantation are useful not only for improving therapy, but also in advancing our understanding of the basic biology of differentiated cell types.

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Figure 1

Bovine adrenocortical cells 6 days following transplantation into a PLGA cylinder beneath the kidney capsule of *scid* mice. Transplants showed a separation of cells into an apparently healthy layer next to the kidney, a zone of dying cells in the middle of the cylinder, and a layer of healthy cells next to the capsule. Hematoxylin and eosin stain. Magnification x 75.

Figure 2

Mouse endothelial cells and macrophages within bovine adrenocortical cell transplants. Sections of transplant tissues were incubated with antibodies against the endothelial cell marker PECAM1 (**a, b**) or the macrophage marker F4/80 (**c, d**). Antibody binding was visualized with fluorescein. **a** and **c** are from animals sacrificed 2 days after transplantation and **b** and **d** are at 6 days. Photographed with triple red/green/blue emission filter; red blood cells show yellow or orange fluorescence, whereas fluorescein appears as pale green, staining cells lining the intercellular spaces in **b** and showing cytoplasmic staining of cells in **d**. The same sections were counterstained with DAPI to show nuclei (**a'** - **d'**). Magnification (a), x 150; (b), x 300; (c, d) x 350.

Figure 3

Bovine adrenocortical cells undergoing apoptosis 24 hours following transplantation. These cells are in the middle layer of the transplant. Apoptotic nuclei were visualized by ligation of a biotin- labeled hairpin oligonucleotide probe, followed by attachment of fluorescein-avidin (**a**). **b** shows nuclei counterstained with DAPI. Magnification x 175.

Figure 4

Double labeling techniques used to investigate patterns of cell death and cell proliferation in bovine adrenocortical cells, illustrated for cells 2 days following transplantation. (**a**) Apoptotic cells in the upper layers of the tissue were labeled by ligation of a hairpin oligonucleotide probe (visualized with Texas Red); sections were then incubated with an antibody against p21 (visualized with fluorescein). Photographed with double red/green emission filter; red blood cells exhibit red autofluorescence but are smaller than nuclei stained red by ligation of oligonucleotide probe. The section shows a rare double-stained nucleus cell that appears yellow. (**a'**) Same section counterstained

with DAPI. (**b**) BrdU was administered to animals 24 hours before sacrifice; BrdU-labeled cells in the basal layer were visualized with an anti-BrdU antibody (red) followed by labeling of cells for the proliferation marker Ki-67 (green). Also photographed with double red/green emission filter. (**b'**) Same section counterstained with DAPI. (**c**) Double labeling for Ki-67 and the cell cycle inhibitor p21. Cells in the basal layer were stained with anti-Ki-67, which was visualized with fluorescein, and with anti-p21, which was visualized with Texas Red. The section was photographed separately with blue and green illumination and the two images were then superimposed. Double-labeled nuclei appear orange; the brighter yellow objects are autofluorescing red blood cells. (**c'**) Same section counterstained with DAPI. Magnification (a), x 225; (b), x 125; (c), x 175.

Figure 5

Comparison of patterns of apoptosis with staining for Ki-67 and p21. Bovine adrenocortical cells were transplanted into *scid* mice and the tissue was harvested for analysis after 6 days. Following staining for apoptosis (ligation of hairpin oligonucleotide probe and visualization with Texas Red), the tissue was labeled with antibodies against the proliferation marker Ki-67 or the cell cycle inhibitor p21 (visualized with fluorescein). (**a, b**) Ligation of hairpin oligonucleotide probe and staining for Ki-67; upper layer of the tissue (**a**) and basal layer (**b**). (**a', b'**) Sections counterstained with DAPI. (**c, d**) Ligation of hairpin oligonucleotide probe and staining for p21; upper layer of the tissue (**c**) and basal layer (**d**). (**c', d'**) Sections counterstained with DAPI. Photographed with dual red/green emission filter; red blood cells exhibit red autofluorescence but are smaller than nuclei stained red by ligation of oligonucleotide probe. Magnification x 175.

Figure 6

Proliferation in transplanted bovine adrenocortical cells. BrdU was administered to animals at 1, 2, or 4 days and the animals were sacrificed at 2 or 6 days (1 -- > 2, 2 -- > 6, 4 -- > 6). Incorporation of BrdU and expression of Ki-67 was assessed by a double-labeling procedure as described in the text. **A** shows the average percentage of labeled cells, ±S.E.; **B** shows the percentages of the cells are Ki-67$^+$, giving this value separately for two subpopulations: those that are BrdU$^+$ and those that are BrdU$^-$.

Figure 7

Levels of nuclear p21 in transplanted bovine adrenocortical cells. The bars show

the percentages of cells which are p21+ when assessed by the more sensitive peroxidase staining technique (light microscopy). The darker hatched bars show the levels of nuclear p21 assessed by avidin-fluorescein labeling and counting by fluorescence microscopy. 1 = tissues harvested 24 to 48 hours following cell transplantation. 6(a) and 6(b) = tissues harvested at 6 days, divided into those with PECAM1 staining throughout the basal layer (6(a)) and those with PECAM1 staining at the boundary with the kidney only (6(b)) (see text for further details). Average values ±S.E. On the right the different sensitivity of the peroxidase and fluorescence detection techniques is illustrated. An aliquot of the suspension of bovine adrenocortical cells used for transplantation was pelleted, then processed in the same manner as transplant tissues. (**a**), section of the cell pellet stained with anti-p21 and visualized by peroxidase staining; (**b**), section stained with the same antibody and visualized with avidin-fluorescein. Magnification x 100.

Figure 8

Relationship between proliferation and p21 in transplanted bovine adrenocortical cells. The three groups are as described in the legend to Figure 7. A shows the percentage of cells labeled by an anti-Ki-67 antibody, ±S.E. B shows the percentages of the Ki-67^{+} and Ki-67^{-} populations that were also stained by the anti-p21 antibody. For both A and B the secondary antibodies were visualized using avidin-fluorochrome conjugates.

Figure 9

A transplanted bovine adrenocortical cell in mitosis showing both Ki-67 (green) and p21 (red), photographed with a double red/green emission filter; the lower image shows the same image overlaid with the blue fluorescent image resulting from counterstaining of the section with DAPI to visualize nuclei and chromatin. Magnification x 800.